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REGULATION OF THE ELECTROPHYSIOLOGICAL PROPERTIES OF MATURE BULLFROG SYMPATHETIC GANGLION NEURONES BY NERVE GROWTH FACTOR

BY

PHILIP TRAYNOR

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1988

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ISBN 0-315-45498-9

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TITLE OF THESIS: REGULATION OF THE ELECTROPHYSIOLOGICAL PROPERTIES OF MATURE BULLFROG SYMPATHETIC GANGLIA NEURONES BY NERVE GROWTH FACTOR

DEGREE: MASTER OF SCIENCE
YEAR THIS DEGREE GRANTED: 1988

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Regulation of the electrophysiological properties of mature bullfrog sympathetic ganglia neurones by nerve growth factor" submitted by Philip Traynor in partial fulfillment of the requirements of the degree of Master of Science in Pharmacology.

P.A. Smith
Supervisor

R.S. French

Tessa Gordon

....

....

Date Aug. 15, 1988

TO MY PARENTS, FOR ALL THIER ENCOURAGEMENT
AND SUPPORT THROUGHOUT MY EDUCATION.

ABSTRACT

Transection of the axon (axotomy) of a neurone promotes specific electrophysiological changes in the associated cell body. In amphibian sympathetic neurones, these changes include (1) an increase in spike width, (2) a decrease in afterhyperpolarization duration, (3) and a decrease in afterhyperpolarization amplitude. This suggests that specific membrane properties of neuronal cell bodies are maintained by contact of their terminals with peripheral targets. Furthermore, the interruption of a nerve-target connection may result in a loss of accessibility to trophic factors produced by the target (Czeh et al., 1978; Gordon, 1983; Gallego et al., 1987). This loss of trophic support may therefore initiate the electrophysiological changes seen in axotomized neurones.

To test this possiblity, the effects of Nerve Growth Factor (NGF), a trophic factor responsible for the maintenance and regeneration of peripheral sympathetic and sensory neurones (Cohen et al., 1954), were examined in the paravertebral sympathetic ganglia of *Rana catesbeiana*, using intracellular recording techniques.

This investigation addressed the following questions: (1) Do neurones maintained in explant culture (in the absence of NGF) exhibit electrophysiological changes

similar to those seen after axotomy in vivo ? (2) Does exogenously applied NGF in culture medium prevent the electrophysiological response to axotomy and target removal? (3) Does the inclusion of NGF antibodies (Anti-NGF) in culture medium enhance the electrophysiological effects of axotomy and target removal? (4) Does the length of the severed axon influence the electrophysiological effects of axotomy and target removal? (5) Is there a correlation between the injury-induced electrophysiological changes, and the morphological properties of explants maintained in different culture conditions? (6) Is NGF present endogenously within Rana catesbieana?

The following results were obtained: (1) Explanted sympathetic ganglion neurones undergo similar electrophysiological changes to axotomized neurones in vivo. (2) NGF prevents some of the axotomy-induced changes suggesting that it is involved in the maintenance of specific action potential parameters, especially afterhyperpolarization duration. (3) Anti-NGF enhances the electrophysiological changes produced by axotomy, which suggests that endogenous NGF regulates the electrical properties of amphibian sympathetic neurones. (4) The length of the severed axon also determines the magnitude of the electrophysiological response to axotomy. This provides evidence for the retrograde transport of NGF or possibly other unknown trophic substances. (5) The magnitude of spike width and afterhyperpolarization amplitude are correlated with growth (neurite production).

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor and friend Dr. Peter A. Smith, for all his help, enthusiasm, and invaluable guidance throughout this project.

I would also like to thank my co-supervisor Dr. William F. Dryden, for his advice and many helpful discussions concerning this study.

I am greateful to Mr. Darin Brox, for his computer skills and assistance in organizing this thesis into a presentable format.

Special thanks to Ms. Tina Cho and Mr. Greg Morrison who performed much of the graphics, and also to Mrs. Marina Maunder for typing the original manuscript.

I would also like to thank the Alberta Heritage Foundation for Medical Research for thier financial support in the form of a studentship.

Finally, I wish to thank my wife Catherine for her patience, understanding and continuous support throughout my studies.

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LIST OF ABBREVIATIONS

BFSG	Bullfrog Sympathetic Ganglia.
NGF	Nerve Growth Factor.
Anti-NGF	Nerve Growth Factor Antibodies.
AP	Action Potential.
AHP	Afterhyperpolarization.
RMP	Resting Membrane Potential.
I	Current.
E	Voltage.
R	Resistance.
IR_{Me}	Voltage drop across a microelectrode.
R_{in}	Input resistance
$M\Omega$	Megaohms.

ms	Milliseconds.
nA	Nanoamperes.
mM	Millimolar.
mV	Millivolts.
h	Hours.
min	Minutes.
cm	Centimeter.
mm	Millimeter.
Ca2+	Calcium ion.
K+	Potassium ion.
Na+	Sodium ion.
Fig	Figure.

Introduction

I. TRANSECTION OF PERIPHERAL NERVE AXONS AND CHROMATOLYSIS.

The transection of a nerve axon is a surgical procedure referred to as axotomy. That portion of the axon separated from the cell body degenerates (Waller, 1850) while the perikaryon which remains attached to the proximal portion of the axon, undergoes a series of characteristic morphological changes (Hyden, 1960, Leiberman, 1971). This reaction to axon-injury was termed chromatolysis by Marinesco (1896). Later technical advances led to the discovery of metabolic (Watson, 1965; 1972 Hendry, 1975) and electrophysiological (Kuno et al. 1974, Purves, 1975, Kelly et al., 1986, Gallego et al., 1987) changes, which accompany the structural alterations occuring in response to axon transection. It has been suggested that these axotomy-induced morphological, biochemical and electrophysiological changes may be associated with the initiation of axonal regeneration towards previously innervated target cells (Gordon, 1983, Gordon et al., 1987; Gurtu and Smith, 1988).

This study concentrates primarily upon the electrophysiological changes which occur as a consequence of axotomy.

II. ELECTROPHYSIOLOGICAL PROPERTIES OF AXOTOMIZED PERIPHERAL NEURONES.

Analysis of the action potential (AP) parameters of axotomized sensory, sympathetic and motor neurones has demonstrated some general trends in the electrophysiological changes produced by axon transection. In the majority of cell types investigated the afterhyperpolarization (AHP) duration and amplitude decreased, while spike width increased (Kuno et al., 1974; Czeh et al., 1978, Kelly et al., 1986; Gallego et al., 1987; Gordon et al 1987., Shapiro et al., 1987 and Gurtu and Smith, 1988). Neverthless, certain cell types were discovered which generated APs with increased AHP durations in response to axotomy (Kuno et al., 1974; Gustafsson and Pinter, 1984). Upon comparision of the measured AP parameters and other electrophysiological properties such as conduction velocity between individual populations of axotomized neurones (example: soleus motor neurones verses medial gastronemius motor neurones, Kuno et al., 1974), it was found that the axon transection-induced electrophysiological changes which occurred, reduced the characteristic electrophysiological differences between the different nerve cell populations (Kuno et al., 1974; Gustafsson and Pinter, 1984; Shapiro et al., 1987). These findings were interpreted in terms of the hypothesis that he observed electrophysiological changes occurring in axotomized neurones may be representative of adedifferentiation process in which neurones switch from a highly specialized state towards a common immature state which is conducive to regeneration and the re-establishment of contact with the denervated target organ (Kuno et al., 1974; Gordon, 1983, Gustatsson and Pinter, 1984; Foehring et al., 1986; Gordon et al., 1987; Shapiro et al., 1987; but see also Gurtu and Smith, 1988).

III. WHY DO THESE ELECTROPHYSIOLOGICAL CHANGES OCCUR AFTER AXON TRANSECTION?

It is possible that the observed electrophysiological response of peripheral nerves to axotomy is a reaction to cellular damage in which the size and intracellular ionic concentrations of the neurone are changed (Gallego et al., 1987). Neverthless, studies of sensory ganglion cells, which are unipolar neurones with a single axon which bifurcates into a centrally-directed process and a peripherally-directed process, do not display metabolic, morphological or electrophysiological manifestations when their centrally directed processes are axotomized. However, chromatolytic and electrophysiological changes are readily observed when the peripheral processes of these neurones are severed (Lieberman, 1969a; , 1969b, 1971; ; Czeh et al., 1977; Gallego et al., 1987). Therefore, the absence of any changes after the transection of the central processes in sensory ganglion cells suggests that cellulardamage is not always the direct cause of the effects of peripheral axotomy. Rather, the interuption of a peripheral process and the resulting loss of continuity between the cell body and peripheral target may mediate the observed changes.

Consequently, these results suggest that certain electrical properties of peripheral nerves are maintained by contact with their peripheral targets. The loss of contact with the target may result in a loss of accessibility to trophic factors, produced by the target (Czeh et al., 1978; Gallego et al., 1987), and consequent initiation of the cell body response to axotomy.

IV. TROPHIC FACTOR REGULATION OF PERIPHERAL NERVES.

Studies of the interaction between motor neurones and muscle tissue in adults have provided evidence which points to the existence of one or more trophic factor(s) which may be retrogradely transported through the motor axon from the muscle and which may be responsible for the maintenance of the normal electrophysiological properties of motoneurones (Czeh et al., 1978). Since the nature of these factors remains unknown, studies of the trophic factor dependence of motorneurones can only involve indirect methods of investigation.

In contrast, a good deal of evidence supports the hypothesis that Nerve Growth Factor (NGF) may play an important role in the maintenance, axonal sprouting and regeneration of sympathetic and sensory ganglionic neurones in the periphery (Levi-Montalcini and Angeletti, 1968) as well as cholinergic neurones (Hefti and Weiner, 1986) in the CNS. The detection of NGF occurred unexpectedly as a result of experiments performed in order to investigate whether the volume of peripheral tissues determines the degree of development of the innervating spinal motor and sensory systems (Bueker, 1948; see also Hamburger, 1934). Transplantation of mouse sarcoma tissue into the body wall of young chick embryos induced a significant increase in the size of the sensory ganglia supplying the area of the tumour transplants. However, it was Levi-Montalcini and Hamburger, in 1951, who observed that embryos bearing transplants of mouse sarcomas " 37 " and " 180 " not only had enlarged sensory ganglia, but the sympathetic ganglia immediately adjacent to the tumour and also those remote from it where enlarged (Levi-Montacini and Hamberger, 1951). They suggested that the sarcoma tissue released a diffusible factor into the circulation that was responsible for the observed effects. The most convincing evidence for the existence of a diffusible factor was the co-cultivation of sarcoma tissue with sensory and sympathetic ganglia, which produced an impressive outgrowth of nerve fibres from the ganglionic explants (Levi-Montalcini et al., 1954).Induction of fiber outgrowth from chick sensory ganglia is still the most commonly used bioassay for NGF or NGF-like activity.

Cohen (1960) partially purified NGF from the submaxillary gland of the mouse, which subsequently led to the development of NGF-antibodies. Destruction of the peripheral sympathetic system by NGF antibodies, provided strong evidence for a physiological role for NGF (Cohen, 1960).

Since NGF is an important growth regulator of the peripheral nervous system, a widely accepted hypothesis states that NGF is ubiquitous and should be detectable in most tissues (Murphy et al., 1984). This hypothesis is supported by the fact that peripheral target organs grown in culture secrete proteins into the medium which react with NGF antibodies and are active in NGF biological assays (Pantazis et al., 1977; Lango, 1978). Furthremore, NGF has been detected *in vivo* in several peripheral targets (Korsching and Thoenen, 1985) and treatment of sympathetic ganglia with colchicine or 6-hydroxydopamine, results in a decrease of NGF concentration in ganglion cell bodies and a corresponding increase in peripheral target organ NGF concentration. Therefore, this suggests nerve cell uptake of target derived NGF (Korsching and Thoenen, 1985).

NGF is retrogradely transported by peripheral sympathetic and sensory neurones from peripheral targets towards the cell body (Korsching and Thoenen, 1985). . This transport process is believed to occur throughout the lifetime of the nerve cell (Hendry et al., 1974; Levi et al., 1980). Inhibition of the uptake and subsequent retrograde transport of NGF following 6-hydroxydopamine or NGF antibody treatment, causes destruction of major parts of the sympathetic and/sensory nervous systems in developing or newborn animals, and produces changes similar to those produced by axotomy in adult animals (Thoenen , 1968; Levi-montalcini, 1978). Of particular interest is that the cell death (in neonates) and the metabolic changes in the cell body produced by 6-hydroxydopamine and axotomy can be prevented by exogenous application of NGF (Hendry et al., 1974; Levi-Montalcini, 1975). Due to their dependence on NGF, sympathetic ganglia exhibit several advantages for studying the regulation of peripheral nerve electrophysiological properties by trophic factors: Firstly, the presence of NGF has been detected in several in vitro preparations of sympathetically-innervated target organs (Thoenen and Barde,1980; Murphy et al., 1984). Secondly, the selective uptake and retrograde axonal transport of NGF has been demonstrated in paravertebral sympathetic ganglia (Hendry et al., 1974; Korsching and Thoenen, 1985). Thirdly, the induction of fiber outgrowth from sympathetic ganglia is an effective bioassay for determining the tropic activity and presence of NGF. Lastly, only limited electrophysiological studies have previously been undertaken in order to investigate the dependency of specific AP parameters of sympathetic ganglion neurones on NGF accessibility. Consequently, the results obtained from studies on this model system, may provide information which may help to explain the electrophysiological response of peripheral nerves to axon section.

V. RATIONALE FOR THE UTILIZATION OF BULLFROG SYMPATHETIC GANGLIA.

In several cell types previously studied the generalized electrophysiological response to axotomy included a depression of synaptic transmission between preganglionic and chromatolyzed post-ganglionic neurones (Eccles et al., 1958; Purves, 1975; Mendell et al., 1976). These changes in the electrical properties of peripheral nerves as a consequence of axotomy are thought to be due to changes in the dendritic morphology of the postganglionic neurones. Postganglionic dendritic morphological alterations result in a subsequent detachment of preganglionic fibres, (Lieberman, 1971; Matthews and Nelson, 1975; Purves, 1975). Therefore, in order to eliminate the possibility that the injury-induced changes in dendritic arborization are responsible for the observed alterations in AP parameters, the effects of axotomy on bullfrog (Rana catesbeiana) paravertebral sympathetic ganglia were studied. The post-ganglionic neurones of bullfrog sympathetic ganglia (BFSG) do not possess dendrites, and all preganglionic fibre synaptic contacts are made directly with the axon hillock and cell body of post-ganglionic fibres (Weitsen and Weight; 1977, Baluk, 1986; Gordon et al., 1987). Consequently, axotomy of these neurones should result in electrophysiological changes representative of the membrane response to axotomy rather than changes in dendritic morphology and synaptic connections. Furthermore, axotomy of post-ganglionic bullfrog sympathetic ganglia neurones, produces a series of select and reproducible electrophysiological changes and the ion channels regulating certain AP parameters have been previously documented (Adams et al., 1982b; MacDermott and Weight 1982; Pennefather et al., 1985; kelly et al., 1986; Gordon et al., 1987; Goh and Pennefather 1987; Lancaster and Pennefather 1987).

VI. IN-VITRO ELECTROPHYSIOLOGICAL STUDIES OF EXPLANTED BULLFROG SYMPATHETIC GANGLION NEURONES.

This study tested the hypothesis that the electrophysiological changes which occur in BFSG neurones as a consequence of axotomy result from the loss of a retrograde supply of NGF from the target. In order to control the immediate extracellular environment this study was performed exclusively on in vitro preparations of BFSG explants (Gruol et al., 1980). Since, BFSG can be readily maintained in explant culture in the absence of NGF (Gruol et al., 1980), it is an especially useful , adult neuronal preparation for testing this hypothesis. Furthermore, these neurones do not undergo chromatolysis when axotomized (Gordon et al., 1987). Also, absolute control of the extracellular environment ensured that exogenously-applied mouse salivary gland NGF, was not neutralized by the possible production of NGF antibodies as might occur in vivo. The production of such antibodies could prevent any response of the sympathetic ganglionic neurones. Furthermore, in vitro preparations of BFSG resulted in complete isolation from target organs. Such removal of targets prevented in vitro NGF synthesis by target cells and allowed for strict control over the NGF content of the extracellular culture medium.

The first experiment was to test whether neurones growing in explant culture (in the absence of NGF) exhibited electrophysiological changes similar to those seen after axotomy in vivo. The second experiment was to test whether the inclusion of exogenous NGF in explant culture medium reverses the electrophysiological changes produced by axotomy and target removal. If this was the case, it would support the hypothesis that the axotomy-induced electrophysiological changes result from a loss of a retrograde supply of NGF from the previously innervated target organ.

Since certain quantities of endogenous NGF or NGF-like molecules are produced by glial tissue of axotomized peripheral nerves (Assouline, 1987; Taniuchi, 1988), regenerating sympathetic ganglion cells in vitro might have access to a certain unknown amount of NGF or NGF-like growth promoting molecules. Thus the third experiment was to introduce NGF antibodies (Anti-NGF) into the culture medium in order to determine whether the electrophysiological effects of axotomy could be enhanced, as a result of neutralization of endogenous growth promoting molecules within the bullfrog.

Furthermore, since NGF is retrogradely transported from the target organ toward the cell body via transport systems within the axon (Hendry et al., 1974 ; Levi et al., 1980), the electrical properties of explants in which the BFSG remained attached via the rami communicantes to a section of spinal nerve containing the axotomized sympathetic ganglion neurones were also examined. If NGF is retrogradely transported, the NGF remaining within the axon immediately after axotomy may delay the axotomy-induced electrophysiological changes, or the changes produced would not be as dramatic as those recorded from explanted ganglia in which the rami communicantes have been severed and the spinal nerve completely removed.

Lastly, the electrophysiological changes were compared with morphological changes, to test whether any of the electrophysiological alterations were correlated with growth or the presence of NGF per se. It was also possible to test whether the electrophysiological changes were a simple consequence of chromatolysis or whether their time course was consistent with a role in the initiation of the regenerative process (Gordon et al. 1987).

Materials and Methods

I. EXPERIMENTAL ANIMALS

Adult bullfrogs (Rana Catesbeiana) of either sex were used exclusively throughout this study. Animals were ordered from a biological supplier (Anilab, St. Foy, Quebec) and were stored at room temperature in a terarium habitat with running tap water. These bullfrogs were fed locusts (Locusta migratoria) and remained healthy in this habitat indefinately, provided they were fed at regular intervals.

II. ELECTROPHYSIOLOGICAL PREPARATIONS.

A. Control Preparations

All bullfrogs were pithed and pinned to a dissecting board in the supine position. The skin and rectus abdominis muscle were removed to expose both the thoracic and abdominal cavities. All major internal organs were then removed. During the eviceration, a special effort was made not to damage the paravertebral sympathetic chain or the aorta. Damage to the aorta resulted in extensive bleeding which decreased visibility, and made further dissection difficult. After the eviceration, the body cavity was rinsed and filled with Frog Ringer's solution (table 1), in order to prevent dessication of the paravertebral

TABLE 1

NORMAL FROG RINGER'S SOLUTION

Constituents	Concentration (mM)
NaCl	100.0
KCl	2.0
$CaCl_2 2H_2O$	1.8
TRIS HCl (pH7.2)	16.0
D-Glucose	5.6

sympathetic chain during the remainder of the dissection.

Removal of the VIIth to Xth paravertebral sympathetic ganglia was now completed with the aid of a dissecting microscope (Wild M3), (refer to Figs. 1a and 1b). Fine surgical forceps were used to manipulate only the connective tissue surrounding the ganglia. Once the rami communicantes connecting the ganglia to their respective spinal nerves were cut, and connective tissue partially removed, the ganglia were gently transferred into a 35mm petri dish containing chilled Ringer's solution.

Excess connective tissue was removed from the ganglia under 40x's magnification. Ganglia were then bathed in 1% collagenase (type 1A, Sigma) for 20 minutes. The collagenase was then removed, and the ganglia pinned firmly with fine stainless steel insect pins (Minuten Pins, Fine Science Tools) to transparent silicone rubber (Sylgard, Dow Corning Corp.) at the base of the 35mm petri dish by the remaining connective tissue. The preparation was bathed in standard culture medium and was now ready for intracellular recording.

B. Preparation of cultured neurones

1. Preparation of explant cultures:

The preparation of BFSG explants utilized in this study was a modification of the method developed by Gruol et al., (1981). The entire dissection and culture procedure was performed within the confines of a Laminar flow hood (Pure-aire) and aseptic techiniques were used in order to prevent contamination of explant cultures.

Adult bullfrogs were pithed and pinned to a dissecting board. 9 gram/100ml iodine was applied to the ventral portion of the body and the skin and rectus abdominus muscle were removed. The animal was then carefully evicerated so as to expose the paravertebral sympathetic nerve chains. The thoracic and abdominal cavities were rinsed with sterile Frog Ringer's solution. Following this, the VIIth to Xth paravertebral sympathetic ganglia were removed under a dissecting microscope,(Figs. 1a and b) and placed in a Ringer's-collagenase (1mg/ml) (type 1A, sigma) solution, which was sterilized by filtration through 0.2 micrometer Millex-GV filtration units (Millipore Corp.) and shaken occassionally. The capsular material and connective tissue surrounding the ganglia were dissected away and each individual ganglion was cut into 4 pieces.

35mm tissue culture dishes (Nunc, Denmark) were coated with 0.2 ml gelatin (Knox, Canada) and left to dry for 30 min. Fibrinogen (4mg/ml) (Sigma) was dissolved in complete

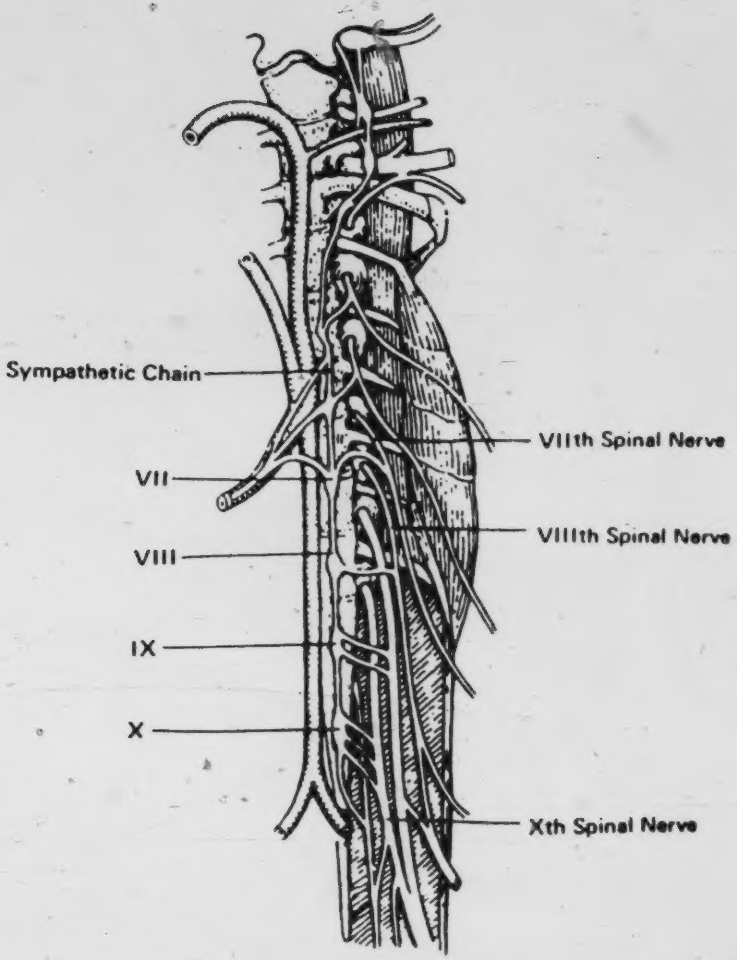

Sympathetic Chain
VIIth Spinal Nerve
VII
VIIIth Spinal Nerve
VIII
IX
X
Xth Spinal Nerve

FIGURE 1A : Anatomy and Organization of Frog Paravertebral Sympathetic Ganglion

Skok (1965)

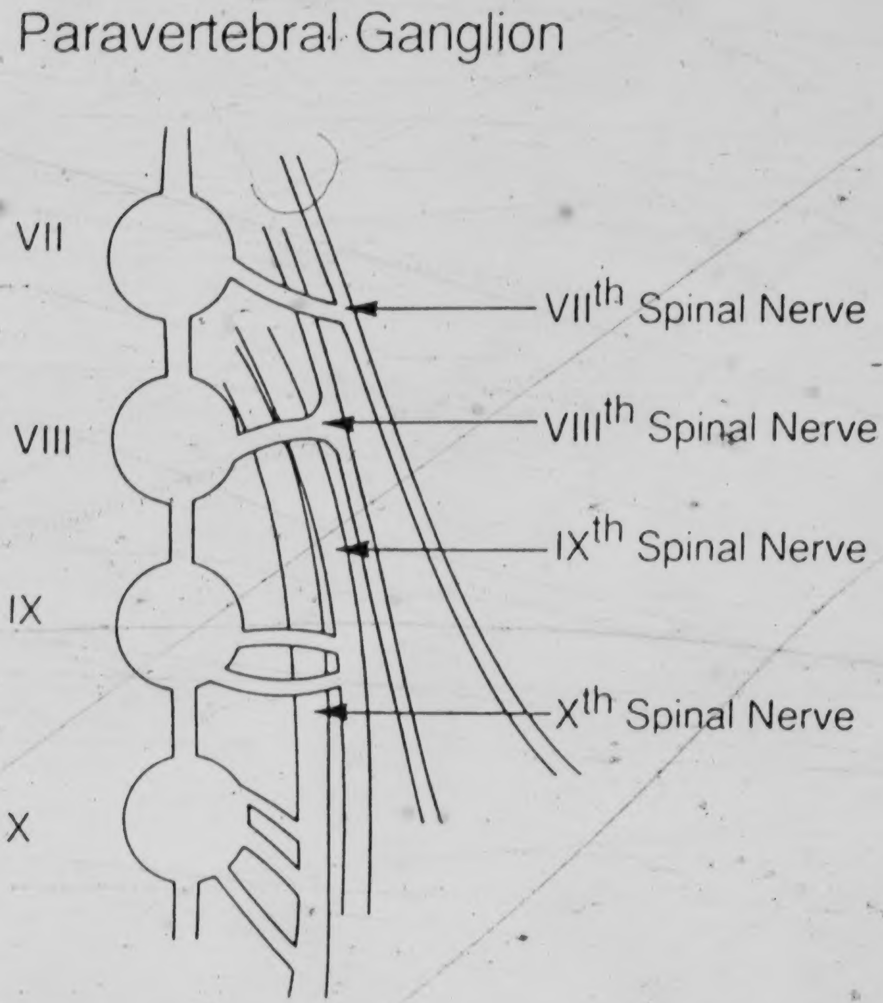

Paravertebral Ganglion
VII
VIII
IX
X
VIIth Spinal Nerve
VIIIth Spinal Nerve
IXth Spinal Nerve
Xth Spinal Nerve

FIGURE 1B :

Schematic Diagram of the VIIth to Xth Paravertebral Sympathetic Ganglia.

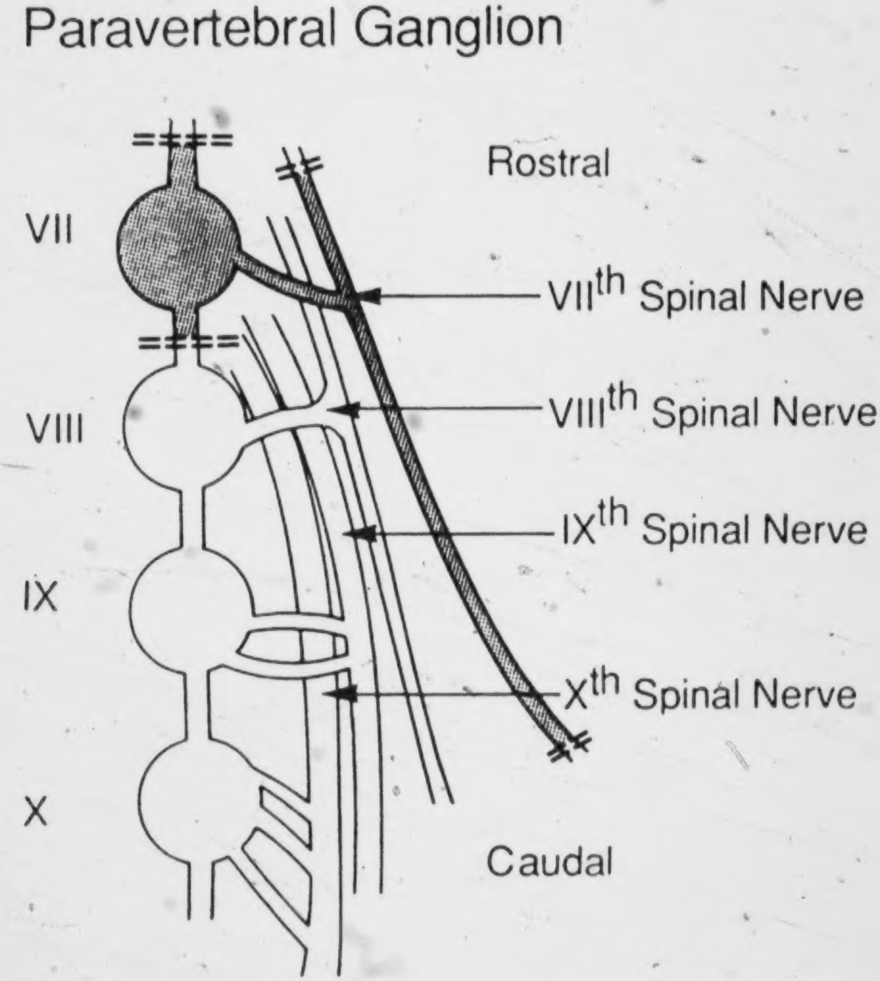
culture medium containing no fetal bovine serum (FBS). This fibrinogen solution was sterilized by filtration through a 0.2micrometer millex-GV filter unit. Two to four drops of fibrinogen solution was placed in each 35mm gelatin-coated tissue culture dish so as to form a smooth film on the gelatin.

One piece of ganglion was placed within the fibrinogen solution in each culture dish using aseptic precautions. Thrombin (Sigma 1 drop, 2.75 mg/ml) was then added to the dish after filtration through a 0.2 micrometer millex-GV filtration unit. The thrombin reacted with the fibrinogen in the dish to form a fibrin clot over the ganglia, (Harrison, 1907; Carrel, 1912). Once the ganglia were stabilized on the bottom of the petri dish by the clot, complete culture medium plus FBS was then added to each dish.

The culture medium in which the BFSG were maintained were changed once per week, and cultures were maintained for durations up to one month.

2. Preparations of explant cultures with attached spinal nerves

Experimental preparations for this study involved explant cultures of BFSG in which the VIIth to Xth ganglia remained attached via rami communicantes to the VIIth to Xth spinal nerves which contained the severed post-ganglionic axons (plate 1). The method for preparation of this type of explant was similar to that used for preparing ganglion explants except that the dissection procedure was slightly modified. In this dissection, the connective tissue surrounding each of the VIIth to Xth ganglia and spinal nerves was dissected away. Spinal nerves were then cut as far caudally and rostrally as possible, (Fig. 2). Each ganglion together with its corresponding spinal nerve was then removed from the animal and placed in chilled sterile frog Ringer's solution. During removal of the ganglion and attached spinal nerve from the animal, special care was taken to avoid stretching the ramus communicans which attach the spinal nerve to the ganglion, as this can damage the delicate post-ganglionic axons. Also, ganglia were not cut into pieces for these experiments. For the preparation of these explants, it was necessary to attach the spinal nerve to the base of the dish with a fibrin clot. Failure to do this allowed the spinal nerve to float freely in the culture medium, which could prevent adherence of the ganglion to the base of the dish and therefore make intracellular recording difficult.



Paravertebral Ganglion
Rostral
VII
VII^th Spinal Nerve
VIII
VIII^th Spinal Nerve
IX^th Spinal Nerve
IX
X^th Spinal Nerve
X
Caudal

FIGURE 2 :
Dissection of Ganglion with Spinal Nerves
Remaining Attached.

▨ Denotes portion of Sympathetic trunk and Spinal Nerve Removed.

==== Denotes location of Surgical Section.

III. Preparation of Culture Medium

The medium utilized in this study is a modification of the medium developed by McMahan and Kuffler (1971). Gruol et al.,(1981), have demonstrated the effectiveness of this medium for the maintanence of explant cultures of BFSG for extended periods of time.

A. Preparation of standard culture medium

Liebovitz L-15 medium (Gibco) (table 2) was diluted to 80%, as this is the concentration of medium suitable for amphibian cell cultures. The L-15 medium was supplemented with 1mM $CaCl_2$, 10mM glucose, 100 U/ml penicillin, 100 mg ml streptomycin and 0.8 mM glutamine. Culture medium was prepared in 500ml quantities, using deionised water. The pH of the medium was adjusted to pH 7.2 by the addition of Hepes acid or NaOH as required. This solution was sterilized by filtration through 0.2 um Nalgene filtration units (Nalge Co).

The complete culture medium consisted of 93% of the above medium plus 0.3% methylcellulose and 7% fetal bovine serum (FBS) (Gruol et al, 1981). The dry methycellulose was first autoclaved (120° C for 35min) in 100ml glass culture bottles (Gibco), prior to the addition of 75ml of sterile medium to each bottle containing the sterile methylcellulose. The methylcellulose dissolved in the

TABLE 2

L-15 (Leibovitz) MEDIUM

Constituents	Concentration (mM)
Inorganic salts	
$CaCl_2$	1.26
KCl	5.0
KH_2PO_4	0.4
$MgCl_2$	0.9
$MgSO_4$	1.0
NaCl	137.0
Na_2HPO_4	1.34
Other components	
D(+) Galactose	5.5
Phenol Red	10.0 mg/ml
Sodium pyruvate	5.0

Amino acids	mM
L-Alanine	4.4
L-Arginine	2.4
L-Asparagine	1.9
L-Glycine	2.4
L-cysteine	0.9
L-glutamine	1.8
L-Histidine	1.4
L-Isoleucine	1.7
L-Lycine	0.45
L-Methionine	1.1
L-Phenylalanine	1.3
L-Serine	1.8
L-Threonine	4.6
L-Tryptophan	0.09
L-Tyrosine	1.5
L-valine	1.5

Vitamins	mg/ml
L-Ca pantothenate	1.0
Choline chloride	1.0
Folic acid	1.0
i-Inositol	2.0
Nicotinamide	1.0
Pyridoxine HCL	1.0
Riboflavin-5-phosphate sodium	0.1
Thiamine monophosphate	1.0

culture medium only after 72 hours of continuous shaking at 4°C. The culture medium was then stored at 4° C in the 100ml glass bottles.

B. Preparation of standard culture medium plus NGF

Explants which received NGF treatment were maintained in standard culture medium plus NGF (50ng/ml). 100 microgram samples of mouse salivary gland 2.5s NGF was obtained from Dr. R. Murphy (Dept. of Anat. and Cell Biol., University of Alberta.). Therefore, dilutions were performed in order to obtain the desired NGF concentration in the standard culture medium.

The available NGF was not isolated under sterile conditions and therefore if it was placed in sterile culture medium, contamination of the culture medium would occur. Further, NGF has long been known to adhere to glass instruments, pipettes and paper (Levi-Montalcini et al, 1968) so special precautions must be taken in order to ensure that NGF is not lost during NGF solution sterilization procedures. In order to obtain standard culture medium with a NGF concentration of 50ng/ml, 100ug of NGF was first dissolved in 10ml of standard culture medium, thus giving 10mls of culture medium having an NGF concentration of 10ug/ml. 10ml of sterile FBS was now filtered through a 0.2 micrometer millex-GV filter unit (millipore Corp) in order to saturate both the syringe and filtration unit with FBS. Following this, the 10ug/ml NGF solution was filtered through the FBS-treated filtration unit, into a sterile 20ml beaker previously coated with FBS.

The sterile 10ug/ml NGF solution was subsequently divided into 1ml aliquots, which were frozen at -22° C as stock solutions. One 1 ml aliquot was then added to 199 ml of standard medium resulting in an NGF concentration of 50ng/ml. Standard culture medium containing 50ng/ml NGF stock solution was stored at 4° C and used as required.

C. Preparation of standard culture medium + anti-NGF.

Anti-NGF (affinity-isolated sheep IgG, produced in response to mouse salivary gland 2.5s NGF injection, was obtained from Dr. R. Murphy (Dept. of Anatomy and Cell Biology, University of Alberta). This anti-NGF was available in a concentration of 10 ug/ml in $NaHCO_3$ buffer. A tissue culture concentration of 0.5 ug/ml was required so subsequent dilutions in standard culture medium were performed. Due to both the isolation of anti-NGF under non-sterile conditions and to the adhesive nature of the protein, anti-NGF was sterilized via a procedure identical to that used for the sterilization of NGF. 0.5 ug/ml stock solutions were stored at -22° C and used as required. Phenol Red indicator in the culture medium indicated that the addition of the $NaHCO_3$ buffer to the culture medium, did not significantly alter the pH.

IV. INCUBATION OF BULLFROG SYMPATHETIC GANGLIA EXPLANT CULTURES:

Explants were placed in 35mm culture dishes which contained 2.0 ml of medium, which varied in composition depending on the experimental condition being studied. Culture media were changed once per week. The explants were incubated in moist glass chambers, above a dilute copper sulfate solution (0.1M) at 22° C.

V. INTRACELLULAR RECORDING TECHNIQUES

A. Recording Chamber

In all experiments, 35 mm petri dishes containing BFSG explants or acutely isolated BFSG, functioned as the recording baths. A transparent plastic collar, which was permanently mounted on the stage of an inverted microscope (Olympus-CK Tokyo) held the petri dishes firmly in position above the objective lens of the microscope. The microscope used in these investigations rested upon a heavy cast iron platform. In turn, the platform was supported by 8 tennis balls which served to limit mechanical vibration. The petri dish recording bath was illuminated from above the microscope stage by a 6 volt D.C. incandescent bulb (Osram, Germany). A copper stand which supported an Ag/AgCl ground electrode (Transidyne instruments) was attached to the transparent plastic collar. The entire volume of the recording chamber was approximately 5ml, and both acute and explanted preparations were bathed in standard culture medium during intracellular recording. The entire recording apparatus, was shielded in a grounded Faraday cage. The recording arrangement is illustrated in Figure 3.

B. Stimulation of Bullfrog sympathetic ganglion neurones.

Standard current-clamp microelectrode techniques were used to study neurones in both explant cultures and in acutely-isolated ganglion preparations. Action potentials were produced following the injection of brief, depolarizing pulses of current through the recording electrode or by passing hyperpolarizing current, thus initiating an anodal break spike at the offset of the hyperpolarization.

C. Intracellular Recording Electrodes.

Micropipettes were made from glass capillary tubing (WPI 1B120F6, O.D. 1.2mm) which was cut into 10 cm sections and pulled on a horizontal Fredrick Haer Ultrafine Micropipette Puller. The optical switch scale, glass preheat, primary pull, pull delay and coil temperature of the micropipette puller are measured in arbitrary units, which were determined via trial and error following investigation of microelectrode properties. These settings were often changed throughout this study in order to obtain micropipettes with tip resistances ranging between 50 and 150 M Ω . Micropipettes with the sharpest tips (resulting in minimal penetration injury) and the lowest resistance (suitable for current passage) optimized intracellular recording.

Since the WPI glass contained a fine glass fibre to facillitate electrode filling, micropipettes were simply filled by injection of 3M KCl into the base of the tubing. Micropipettes were then suspended, with the tips immersed in distilled water to prevent drying and crystalization of the filling solution. Micropipettes completely filled to the tips only after 45 to 60 mins.

For intracellular recording, the micropipette was

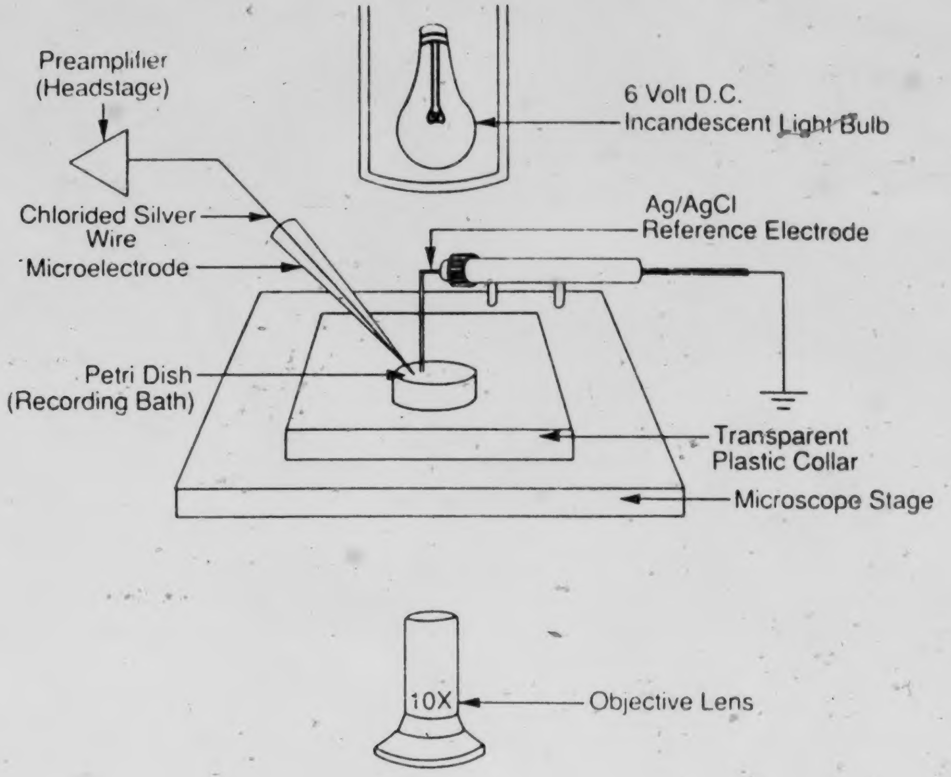

Preamplifier
(Headstage)
6 Volt D.C.
Incandescent Light Bulb
Chlorided Silver Wire
Ag/AgCl
Reference Electrode
Microelectrode
Petri Dish
(Recording Bath)
Transparent
Plastic Collar
Microscope Stage
10X
Objective Lens

FIGURE 3 : Diagram of Recording Chamber used for Intracellular Experiments.

firmly attached to a Narishige MO-103M three dimensional hydraulic micromanipulator, and a chloride-coated silver wire was inserted into the KCl-filled micropipette. The chloride-coated silver wire was directly connected to the recording system described in Fig. 4. The 3M KCl-filled micropipette therefore serves as a salt bridge which conveys electrical information between neurones and the recording apparatus. The silver wire was coated with chloride using a D.C power source. A silver wire was connected to the anode of the D.C. power source while another silver wire was connected to the cathode. Both wires were then immersed in dilute HCl and the power source switched on for 35 sec. This procedure results in the silver wire attached to the anode being uniformly covered with a thin, grey-brown chloride coating.

A silver chloride wire was used within the micropipette so as to obtain a stable half-cell potential that would not drift during an experiment and would not be altered by the passage of small currents. These conditions can only be met by a reversible electrode. Thus in a chloride coated silver electrode the reversible reaction is $Ag + Cl^{-} \quad AgCl + e^{-}$. The function of the silver chloride is to provide a stock of Cl^{-} ions should the reaction proceed to the left (Purves 1981).

FIGURE 4 : Block Diagram of the Electronic Apparatus used for Intracellular Recording.

LEGEND :

(1) AXOPROBE 1, MULTIPURPOSE MICROELECTRODE AMPLIFIER.

(2) DIGITIMER ISOLATED STIMULATORS MODEL DS2

(3) TEKTRONIX 2330 DIGITAL STORAGE OSCILLOSCOPE

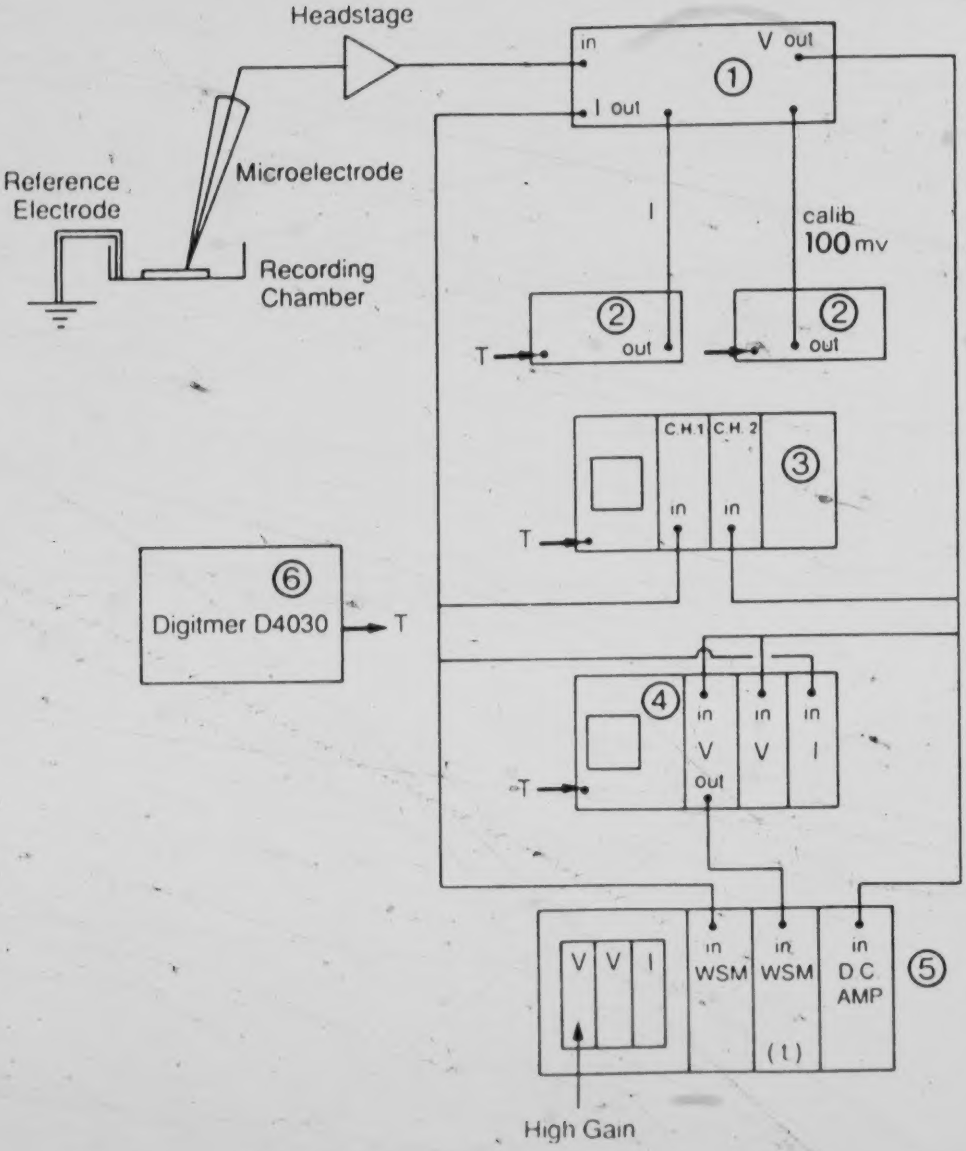
(4) TEKTRONIX 5111A STORAGE OSCILLOSCOPE

(5) GOULD 2400S PEN RECORDER
    i) D.C. amplifier
    ii) waveform storage amplifier

(6) DIGITIMER D4030

(T) TRIGGERED BY DIGITIMER D4030

(t) TRIGGERED BY ISOLATED STIMULATOR-CALIBRATOR


Headstage
Reference Electrode
Microelectrode
Recording Chamber
in
V out
①
I out
I
calib
100 mv
②
out
②
out
T
C.H.1
C.H.2
③
in
in
T
⑥
Digitmer D4030
T
④
in
in
in
V
V
I
out
T
V
V
I
in
WSM
in
WSM
(t)
in
D C
AMP
⑤
High Gain

D. Cell penetration.

1. Offset potential.

A microelectrode was moved into position above the recording chamber containing the BFSG neurones and into the field of view of the microscope (magnification: 150 x). The microelectrode was then lowered until it was submerged in the culture medium bathing the ganglonic neurones. An offset potential which is composed of the liquid junction potential and the tip potential was then recorded. The magnitude of the liquid junction potential is determined by the nature and concentration of the electrolyte solution used to fill the micropipette. The diffusion coefficients of the electrolytes may differ, and due to the concentration gradient that exists on either side of the micropipette, a slight charge separation may occur resulting in a potential difference. K^+ and Cl^- have similar diffusion coefficients and carry a unit charge therefore the tendancy towards change separation is reduced (Purves 1981). Consequently, 3M KCl is often used for salt bridges in electrophysiological studies, due to a reduction of the liquid junction potential and the high concentration decreases the resistance to current flow.

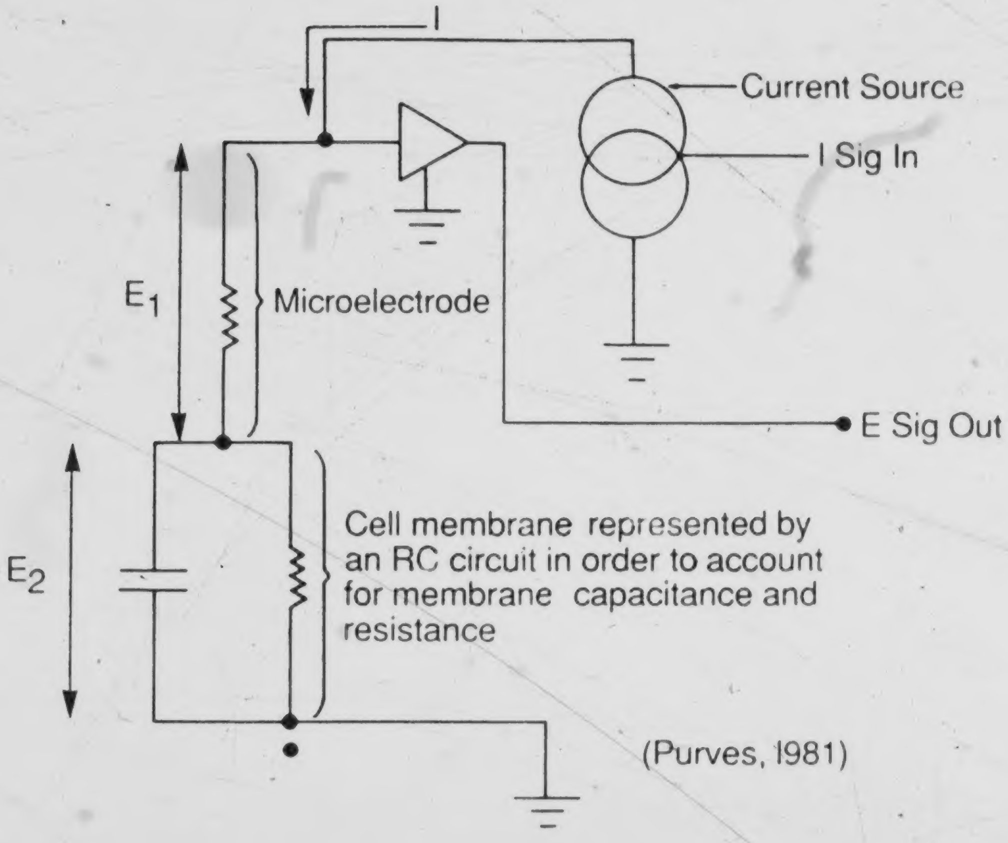
The tip potential is dependent on the physical properties of the microelectrode itself and originates at the interface between the glass of the microelectrode and the electrolyte solution. A charge separation occurs due to the microelectrode glass acquiring a layer of fixed negative charge when in contact with water, which is balanced by a layer of positive charge in the adjancent internal solution. Therefore a potential difference exists across the microelectrode tip. The total offset potential was compensated for using the D.C. offset control in the microelectrode amplifier (Axoprobe-1).

It is important to note that even though the offset potential has been electrically compensated for, during cell penetrations the electrode tip may break thus further altering the tip potential. This change in offset potential cannot be corrected once cell penetration has occured. Therefore the D.C. offset value prior to cell penetration is left unchanged during intracellular recording. Consequently, measurements of absolute membrane potential are subject to a certain degree of error.

2. Balancing microelectrode resistance.

In this study, a single microelectrode simultaneously injected current and measured membrane potential. A problem in this kind of system, is that the voltage drop

FIGURE 5 : CIRCUIT DIAGRAM DISPLAYING THE COMPONENTS OF THE TOTAL VOLTAGE DROP OBSERVED BY THE PREAMPLIFIER.


I
Current Source
I Sig In
E1
Microelectrode
E Sig Out
E2
Cell membrane represented by an RC circuit in order to account for membrane capacitance and resistance

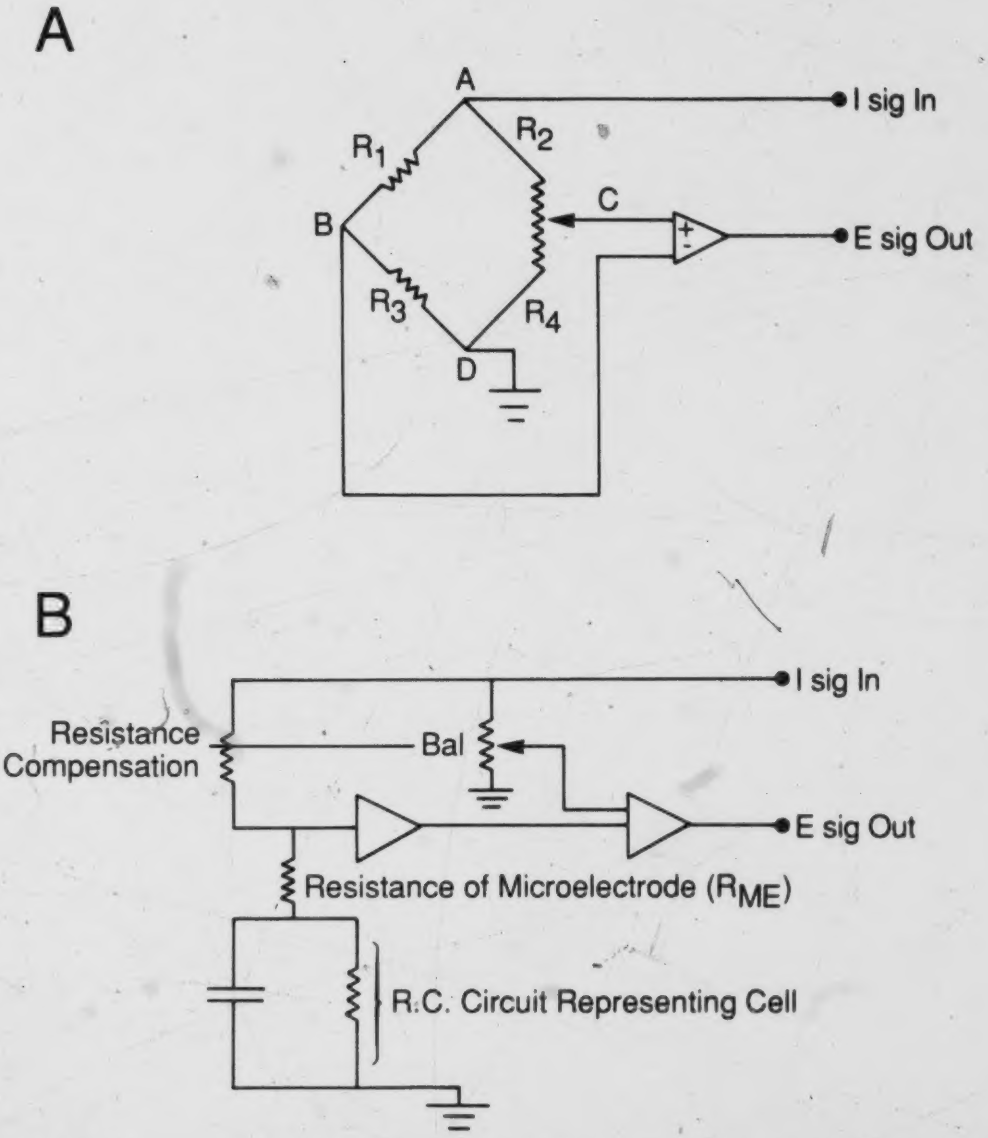
(Purves, 1981)

THE TOTAL SIGNAL E SEEN BY THE PREAMPLIFIER CONSISTS OF THE VOLTAGE DROP E2 ACROSS THE CELL AND THE VOLTAGE DROP E1 ACROSS THE ELECTRODE.
ONLY THE MEASUREMENT E2 IS DESIRED.

FIGURE 6A :
WHEATSTONE BRIDGE CIRCUIT FOR SIMULTANEOUS CURRENT INJECTION AND VOLTAGE RECORDING.

FIGURE 6B :

MODERN CIRCUIT BASED ON THE WHEATSTONE BRIDGE CIRCUIT.


A
A
R1
R2
B
C
R3
R4
D
+
-
I sig In
E sig Out
B
I sig In
Resistance Compensation
Bal
E sig Out
Resistance of Microelectrode (RME)
R.C. Circuit Representing Cell

Resistor R_{ME} and the R.C. Circuit of Figure 6B are equivalent to Resistor R_3 in Figure 6a

observed by the amplifier is the sum of the voltage drop across the electrode (IR_{ME}) and the voltage drop across the cell membrane,(Fig. 5). In the examination of membrane electrical properties, primary interest lies in the changes in membrane potential evoked by the passage of current. The voltage drop across the microelectrode (IR_{ME}) is of no of biological significance and therefore must be removed from the output voltage of the amplifier. This is accomplished using circuit designs based on the Wheatstone bridge circuit (Fig. 6a and b) in which a variable resistor is adjusted using a resistance compensation or bridge control on the amplifier (Axoprobe -1). Adjustment of the bridge control effectively reduces the potential difference across the bridge circuit to zero, and therefore subsequent current injections no longer produce a voltage drop across the electrode.

Therefore, before impalement of a ganglion cell was attempted, the resistance of the microelectrode was balanced using the resistance compensation control on the Axoprobe-1, which also gives a readout of electrode resistance. Thus, current injection produced no voltage response. Since the microelectrode resistance may change as a result of cell penetration, due to breakage of the microelectrode tip, the resistance of the electrode was only roughly balanced prior to cell impalement. Accurate balancing was only attempted after cell penetration.

Once cell penetration was accomplished, and the electrode tip was firmly sealed within the cell membrane, accurate balancing of the electrode resistance was attempted utilizing the method of Engel et al.(1972). The principle of this method is to adjust the resistance compensation (bridge balance) until a point is reached at which the charging of the membrane capacitance at the onset of the injected current pulse is initiated smoothly from the resting membrane potential (RMP).

The unwanted voltage drop across the microelectrode (IR_{ME}) is easily identifiable when the oscilloscope is switched to a high sweep speed (Tektronix 5111A) and the negative capacitance control on the amplifier adjusted for maximal frequency response. The voltage drop across the microelectrode has a characteristic abrupt onset coincident with the current pulse. This is distinguishable from the membrane voltage response which lags in time in response to current injection due to the capacitative properties of the membrane.

Lastly, the stray capacitance of the input circuit must be neutralized using the capacitance neutralization control of the axoprobe-1 amplifier. This stray capacitance, if not accounted for may distort the true membrane voltage response to injected current. The negative capacitance control was set so that a 100mV 20ms calibration pulse appeared completely square on the oscilloscope screen.

3.Techinique for membrane penetration.

Once the microelectrode was positioned within the field of view of the inverted microscope by the micromanipulator, the microelectrode was focused upon and lowered towards the cells of the preparation within the recording chamber. The inverted microscope allowed the individual cells of the ganglion to be distinguished at 150 X's magnification. Therefore, the microelectrode was continually kept in focus and lowered towards a particular cell. Hyperpolarizing current pulses (0.02 nA, 800 ms duration) were passed through the microelectrode every 3.5 s and the bridge balance control adjusted so that no potential drop occured across the electrode. When the microelectrode made contact with the cell surface the alteration in the effective microelectrode resistance caused the bridge to become unbalanced and a potential change could be recorded in response to the current pulse.

With the microelectrode now in contact with the cell surface, the "buzz" button on the axoprobe-1 multipurpose microelectrode amplifier was depressed briefly. Depression of the "buzz" button causes the amplifier to oscillate, this oscillation causes the electrode tip at the cell surface to undergo vibration which increases the probability that the electrode will penetrate and impale the cell.

If impalement was successful, the electrode was often moved slightly further into or out of the cell in order to estabilish a good seal between the cell membrane and the electrode. Cells producing APs with spike heights greater than 70 mV were regarded as having been successfully impaled and therefore suitable for obtaining electrophysiological data.

4. Data Display and Storage.

A permanent record of all amplified signals was obtained through the use of a Gould 2400s Pen Recorder equipped with a D.C. preamplifier and two waveform storage modules, (Fig. 4). The three channels of the pen recorder displayed a current trace and high and low gain voltage traces. The high gain voltage trace was utilized to record all membrane responses while the current trace provided a record of the current injected at any one time. The nominal frequency response of the waveform storage module was D.C. to 10 KHz, with a maximum sampling rate of 100 KHz.

E. Generation of Action potentials

Since BFSG neurones are silent in the absence of presynaptic input, all APs recorded from acute or cultured preparations were evoked as a consequence of direct intracellular stimulation. Direct stimulation of these neurones was acheived by passing a brief depolarizing current pulse through the recording electrode, shortly after successful impalement had occured. Also, anodal break APs were produced as a result of passing a hyperpolarizing current command.

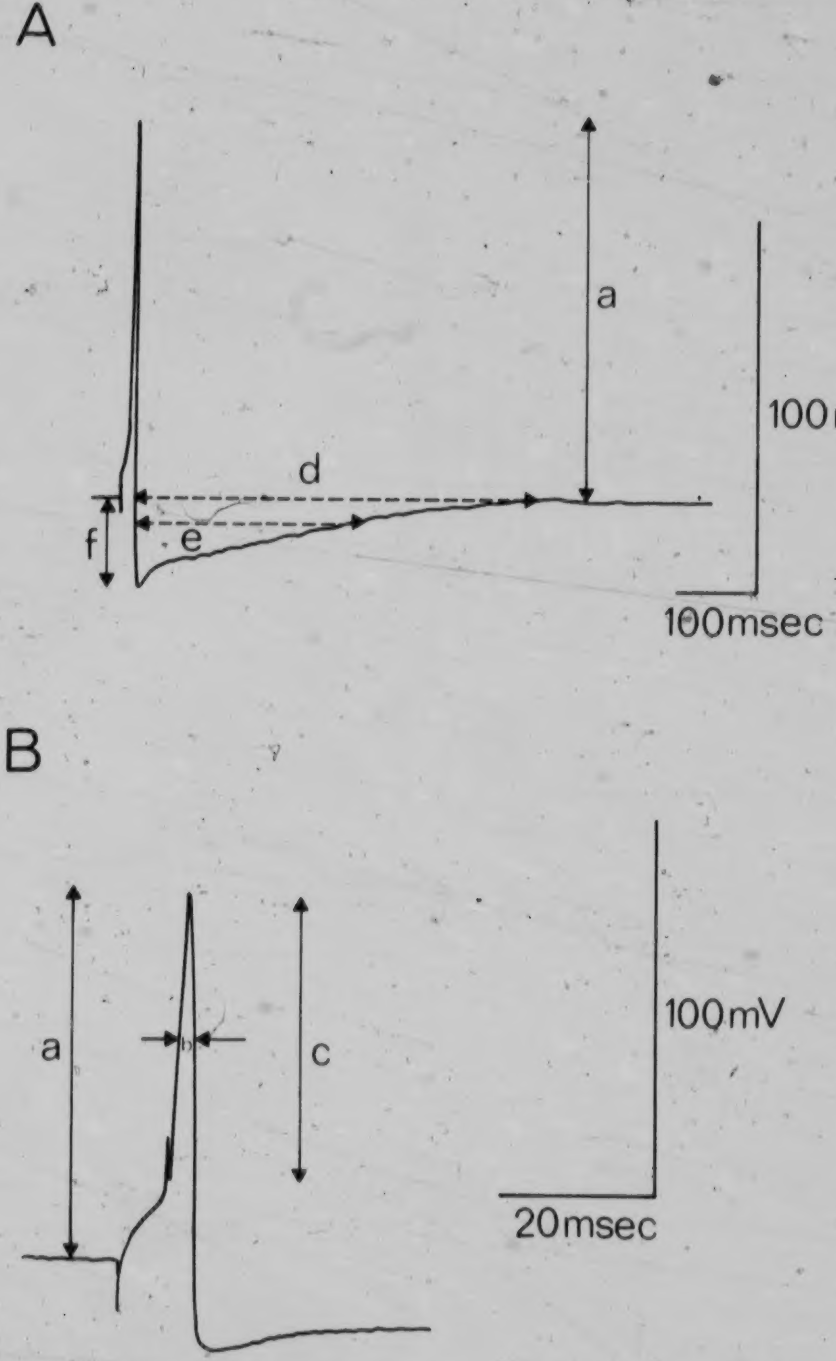
F. Measurement of Action potential Parameters:

In this investigation, the alteration of specific AP parameters was of primary interest. Therefore, measurements of the following parameters were made; (a) spike height from resting membrane potential (RMP) to the peak of the AP, (b) spike duration, measured at 50% of spike height (c) spike height from the inflection point of the AP to the peak of the AP, (d) AHP duration measured from the onset of the hyperpolarization to the point of

FIGURE 7 : MEASUREMENT OF ACTION POTENTAIL PARAMETERS.

a) SPIKE HEIGHT (base)
b) SPIKE DURATION
c) AHP DUATION AT 100% REPOLARIZATION
d) AHP DURATION AT 75% REPOLARIZATION
e) SPIKE HEIGHT (inflection point)
f) AHP AMPLITUDE

Fig. 7



recovery to the RMP, (e) AHP duration measured at 75% of AHP repolarization using the point of linear intersection with the trace as it returns to RMP, (f) AHP amplitude measured from RMP to the peak of the AHP (Fig. 7A and B).

The measurement of AHP duration at 75% of AHP amplitude was made in order to obtain a more reproducible estimate of AHP duration, because this measurement is less subject to error than a measure of AHP duration at 100% repolarization. Although AHP duration at 100% repolarization is more subject to error, this bias would be balanced out due to the large sample sizes obtained.

G. Current-Voltage Curves.

The input resistance of each cell was determined by the construction of a current-voltage curve. This involved the injection of 6, 800-1000ms current pulses of increasing magnitude through a balanced electrode. With each progressively larger current injection, the corresponding voltage response was recorded. A current - voltage graph was then subsequently plotted in which the voltage response to current injection was the dependent variable (y-axis). The input resistance of the cell was then calculated by determining the slope (dE/dI) of the linear portion of the curve in the hyperpolarizing direction.

H. Rheobase Measurement

Rheobase is defined as the threshold current required to just discharge an AP (Gordon et al., 1987), and this measurement can be used to approximate nerve cell excitability. Successive depolarizing current injections of increasing intensity were used to determine the rheobase value of each successfully impaled BFSG neurone.

The value of rheobase could be calculated by simply noting the minimum current value required to produce an AP which was displayed by the Axoprobe-1 microelectrode amplifier. Rheobase could also be determined from permanent records obtained on the Gould 2400s pen recorder. The minimum current required to produce an AP was measured relative to a current calibration pulse.

VI. ELECTRON MICROSCOPY.

The morphological characteristics of BFSG neurone cell bodies were examined in both acute and cultured preparations, using a Philips EM 410 Electron Microscope.

Acutely excised BFSG and BFSG maintained in standard culture medium for 12 days were fixed with glutaraldehyde (2.5%, 15 min.). Ganglia were then dehydrated with ethanol (98%, 15 min.), and propylene dioxide (15 min.). After fixing and dehydration, each ganglion was imbedded in epon for sectioning. A Reichert Ultracut E (diamond knife) was used for sectioning the epon-imbedded ganglia into 80 nanometer sections. Once cut, the individual sections were stained with uranyl acetate and lead citrate and placed on copper-grid discs. These discs were examined under the Philips 410 using a field strength of 60 KV, at magnifications ranging from 1300 x's to 2800 x's.

VII. PHOTOGRAPHY

A Leitz phase contrast inverted microscope with attachable camera and film cassette, was used in order to obtain a photographic record of the effects of changing culture conditions on the morphology of explanted BFSG. For all photomicrographs 400 ASA Ektochrome colour slide film was used, and all film was developed commercially. Low power photomicrographs displaying the explanted ganglia and extending neurites were taken at a magnification of 100 x's, while higher power photomicrographs of neurites were taken at a magnification of 400 x's.

VIII. STATISTICS.

In this study, the two statistical tests utilized were the unpaired Student's t-test and the Duncan's multiple range test. The unpaired Student's t-test was used to determine if a statistically significant difference (i.e. $P < 0.05$) existed between the means of two distinct sample groups of unequal sample size. The Duncan's multiple range test was employed when multiple comparisons were required between the means of several groups of unequal sample size. This test enabled one to determine which pairs of groups were significantly different from each other.

All results are expressed as a mean (x) + standard error (S.E.). In this study, the symbol (n) represents the number of cells present in a particular control or experimental group. All statistics are based on sample sizes of 30 or more.

Results

I. DO THE ELECTROPHYSIOLOGICAL CHANGES OBSERVED IN CULTURE RESEMBLE THOSE OBSERVED AFTER AXOTOMY IN VIVO ?

The in-vivo post-axotomy electrophysiological changes which occur in bullfrog sympathetic ganglia (BFSG) have been extensively studied by Gordon et al.(1986). The major changes in the membrane properties of these neurones were maximized after approximately 14 days post-axotomy. These changes included an obvious decrease in afterhyperpolarization (AHP) amplitude and duration, and an increase in both spike duration and rheobase.

If BFSG neurones maintained in culture undergo electrophysiological changes similar to the in vivo condition, this may suggest that cultured neurones respond via the same cellular mechanisms to axon injury and loss of continuity with their designated targets.

Therefore, axotomized BFSG neurones were maintained as explants in standard culture medium in the absence of target organs for experimental durations of 1 to 30 days, and the time course of the changes in their electrophysiological properties examined.

A. Electrophysiological characteristics of control neurones.

Two neuronal cell types are present in BFSG, these are B - cells and C - cells. These cells can be distinguished both physiologically, in terms of the antidromic conduction velocity of their axons and morphologically on the basis of their cell size, (Nishi et al., 1965; Dodd and Horn, 1983).

Shapiro et al. (1987) have studied the effect of axotomy on the electrophysiological properties of C cells in BFSG neurones and found that in these cells, as in B-cells, AHP amplitude and duration were reduced and spike duration was increased. Since both B and C cells respond similarly to axotomy, no deliberate selection was made with respect to cell size or conduction velocity in this study. Since it is difficult to obtain high quality, long term electrophysiological recordings from c-cells, it is likely that the majority of cells studied in the present experiments were in fact B-cells.

Control records were obtained from acute preparations of BFSG. All control records were obtained within an 8 h period after removal of the ganglia from the animals. APs generated by these neurones were measured with respect to several parameters (refer to methods, Fig. 7), which served as a standard to which all experimental groups were compared. The values for the acute preparations are shown in table 3.

TABLE 3

CHRACTERISTICS OF CONTROL NEURONES.

AHP Duration (100%) Repolarization:	289.5 +/- 11.6 ms
AHP Duration (75%) Repolarization:	178.8+/- 10.8 ms
AHP Amplitude:	17.7 +/- 0.39 mV
Rheobase:	0.165 +/- 0.01 nA
Spike Duration:	2.02 +/- 0.06 ms
Spike Amplitude (Base):	78.11 +/- 0.99 mV
Spike Amplitude (Inflection point):	65.17+/- 1.07mV
Input Resistance:	95.65 M +/- 10.23MΩ
Resting Membrane Potential:	43.8 +/- 0.78 mV

n>60 cells

B. Effects of culture duration on the electrophysiological characteristics of explanted BFSG neurones.

APs were recorded from explanted BFSG in standard culture medium after culture durations of 1-2 days, 5-6 days, 8-10 days, 12-14 days, 16-18 days, 20-22 days, 24-26 days, and 28-30 days. Data from each of these time periods were pooled and are expressed as 1.5 days, 5.5 days, 9 days, 13 days, 17 days, 21 days, 25 days, and 29 days respectively on the graphs presented in Figs. 9 to 17. The use of Duncan's multiple range test showed that several of the AP parameters measured differed significantly between the individual age groups and also in comparision to control cells, while other parameters remained unchanged. Some typical original data records are illustrated in Fig. 8. The changes observed however, bare a strong resemblance to those seen after *in vivo* axotomy (Gordon et al. , 1987).

1. Effects on AHP Parameters:

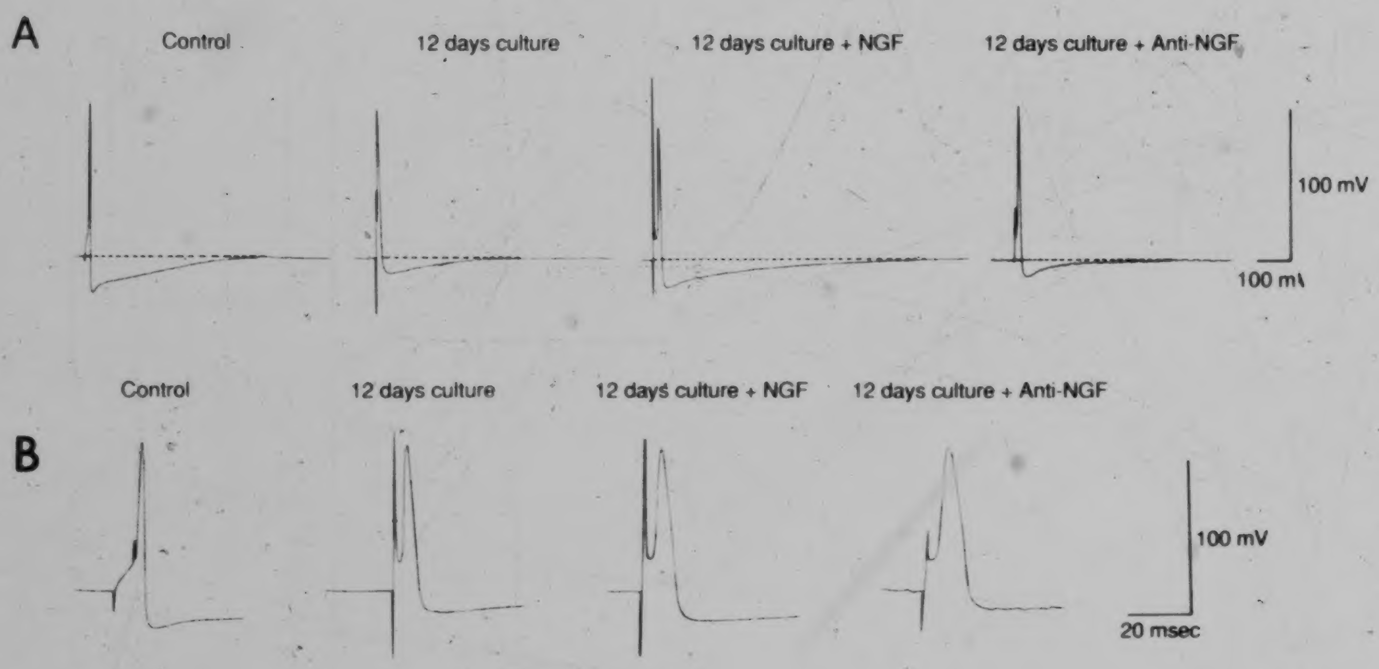
AHP duration at 100% repolarization: A marked decrease in AHP duration at 100% repolarization occured after 1-2 days in-vitro (Figs. 8A and 9). This initial decrease was too rapid to be examined in detail. The AHP contiued to decrease in duration as the time in culture increased, reaching a minimum value of AHP duration after 28-30 days in culture (Fig. 9).

FIGURE 8A :

EFFECTS OF CHANGING CULTURE CONDITIONS ON AHP DURATION AND AMPLITUDE

FIGURE 8B :

EFFECTS OF CHANGING CULTURE CONDITIONS ON SPIKE DURATION


A
Control
12 days culture
12 days culture + NGF
12 days culture + Anti-NGF
100 mV
100 m\
B
Control
12 days culture
12 days culture + NGF
12 days culture + Anti-NGF
100 mV
20 msec

FOR FIGURES 9 TO 17:

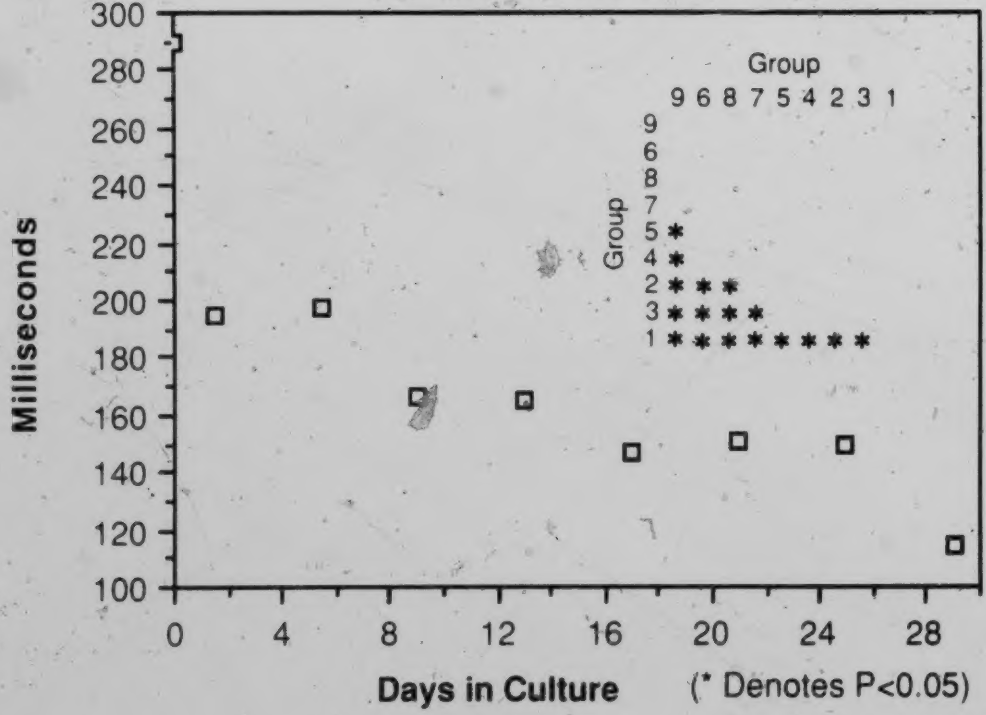
EACH SQUARE REPRESENTS A MEAN VALUE FOR A PARTICULAR PARAMETER MEASURED OVER A GIVEN TIME PERIOD

EXAMPLE: GROUP 2 REFERS TO THE TIME PERIOD 1-2 DAYS.

* DENOTES WHICH TWO GROUPS DIFFER SIGNIFICANTLY FROM EACH OTHER ($p<=0.05$)

Fig. 9 **Afterhyperpolarization Duration 100%**

(symbols represent n>30 cells)



(* Denotes P<0.05)

AHP duration at 75% repolarization: The changes in AHP duration measured at 75% of repolarization were similar to the electrophysiological changes in AHP duration measured at 100%, which occured over the 1-30 day culture period (Fig. 10).

AHP amplitude: AHP amplitude decreased exponentially over the first 10 days in culture (T=7.8 days). This period was followed by a period in which AHP amplitude tended to recover slightly. After 17 days in culture, amplitude then proceeded to decrease, reaching a minimum value after 28-30 days *in vitro* (Figs. 8A and 11).

2. Effect on Spike Duration:

Spike duration measured at 50% of the spike amplitude, (Fig. 7) increased as a function of time in culture. Spike duration (width) increased linearly and rapidly over the first 6 days *in vitro* but appeared to reach a plateau between 8-20 days. The spike width then contuined to increase gradually and still appeared to be increasing after 28 to 30 days *in vitro* (Fig. 8B and 12).

Fig. 10 **Afterhyperpolarization Duration 75%**

(symbols represent n>30 cells)

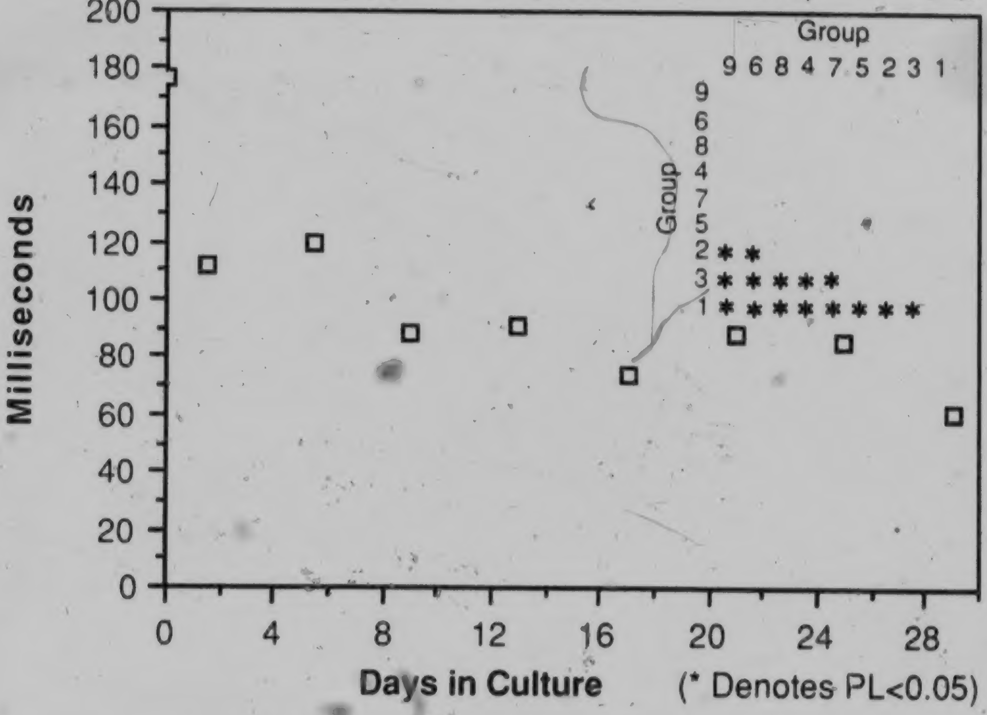


Fig. 11 **Afterhyperpolarization Amplitude**

(symbols represent n>30 cells)

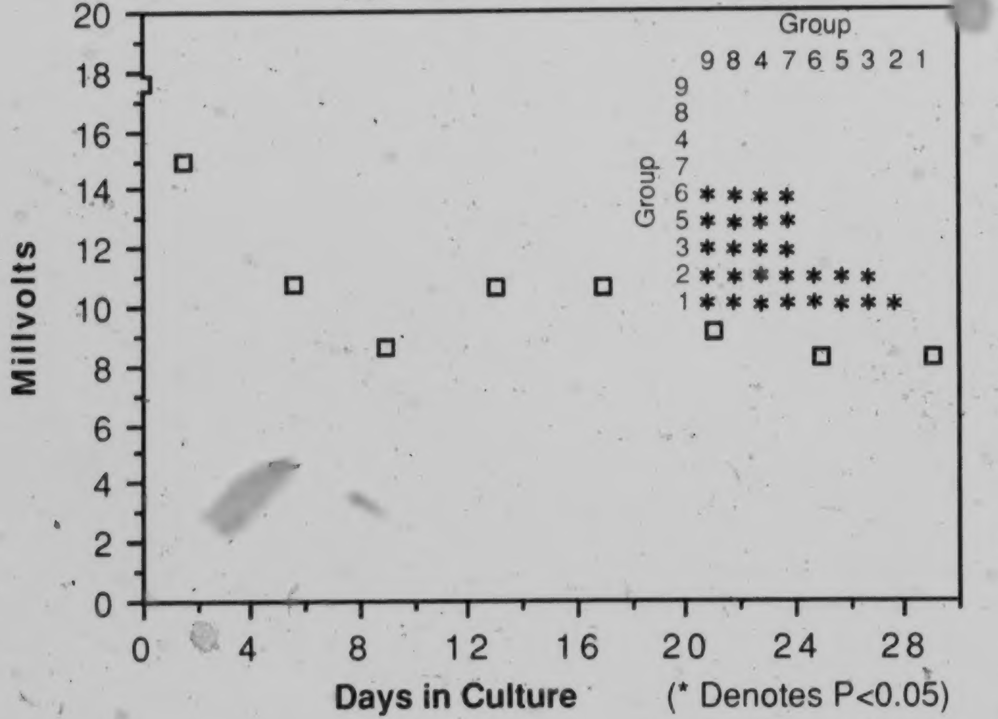
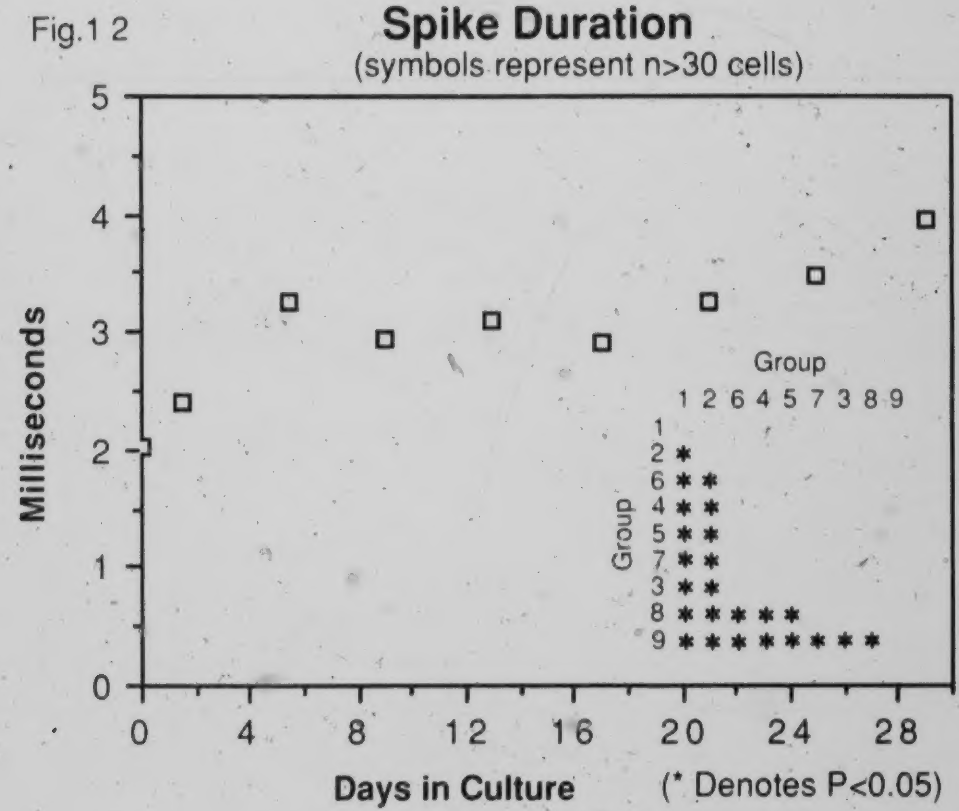


Fig.1 2


Spike Duration
(symbols represent n>30 cells)
Milliseconds
0
1
2
3
4
5
0
4
8
12
16
20
24
28
Days in Culture
(* Denotes P<0.05)
Group
1 2 6 4 5 7 3 8 9
Group
1
2 *
6 * *
4 * *
5 * *
7 * *
3 * *
8 * * * * *
9 * * * * * * * *

3. Effect on rheobase:

There were significant changes in rheobase as the time in culture increased. Rheobase reached a peak value of 0.45 nA after approximately 8-10 days and proceeded to recover back to the initial value of 0.18 nAmps after 28-30 days *in vitro*. There was no significant difference between the mean rheobase values of control cells when compared to the mean rheobase value of cells 28-30 days old (Fig. 13).

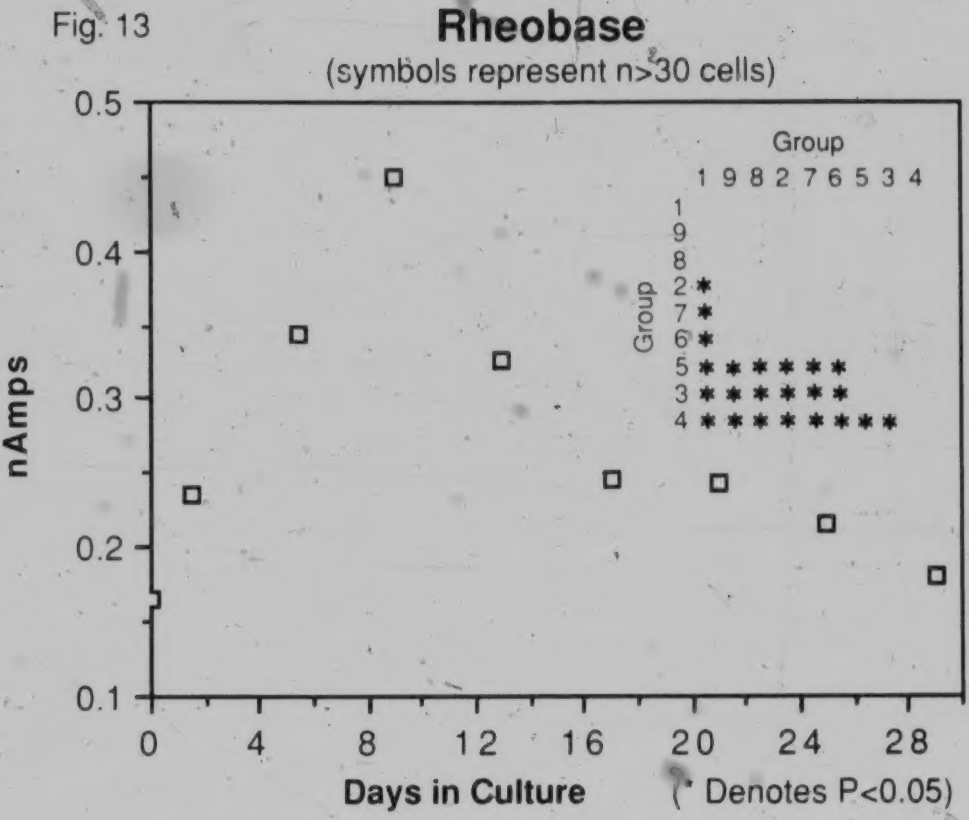
4. Effects on other Parameters:

Resting membrane potential (RMP): Over the 30 day period, it was found that there was relatively little variation in RMP, although a significant decrease was apparent after 1-2 days and after 29 days in culture (Fig. 14).

Input resistance : Input resistance (R_{in}) falls to a minimum value after 12-14 days in culture and a maximum value of 120.6 MΩ was noted after 24-26 days in culture. Neither of these values differed significantly from control. After 28-30 days in culture R_{in} in returned towards the control value (Fig. 15).

Spike amplitude: Spike amplitude measured from both the baseline and from the inflection point remained relatively constant for most of the time *in vitro*. Only after 28-30 days in culture was there a significant decrease in spike

Fig. 13



Group	1	9	8	2	7	6	5	3	4
1									
9									
8									
2	*								
7	*								
6	*								
5	*	*	*	*	*	*			
3	*	*	*	*	*	*			
4	*	*	*	*	*	*	*	*	

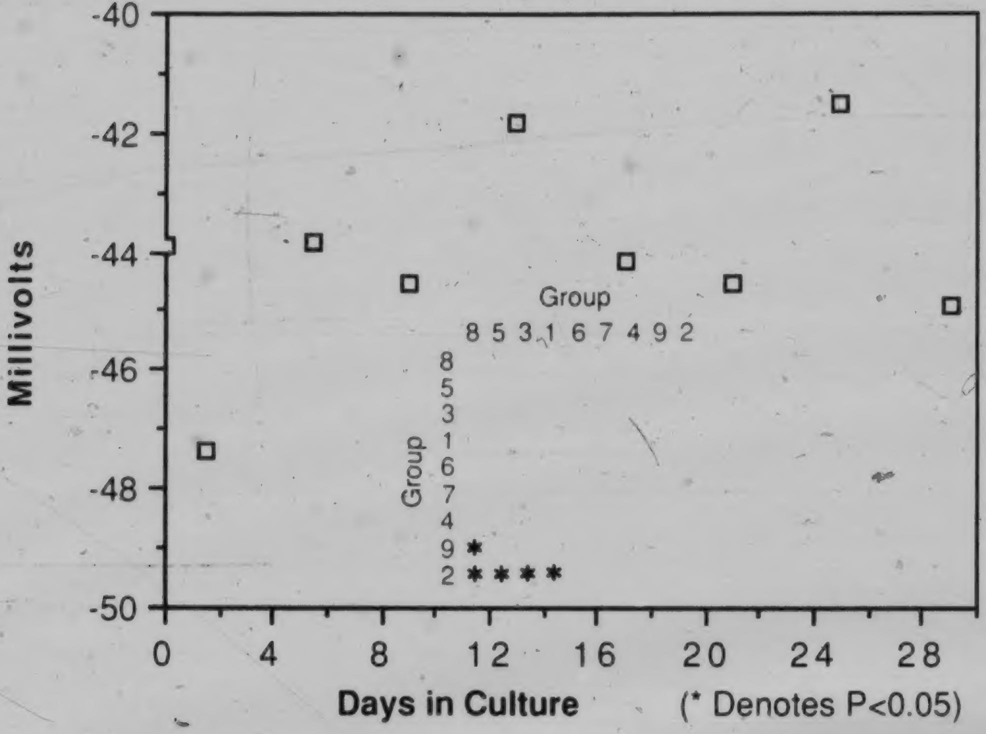
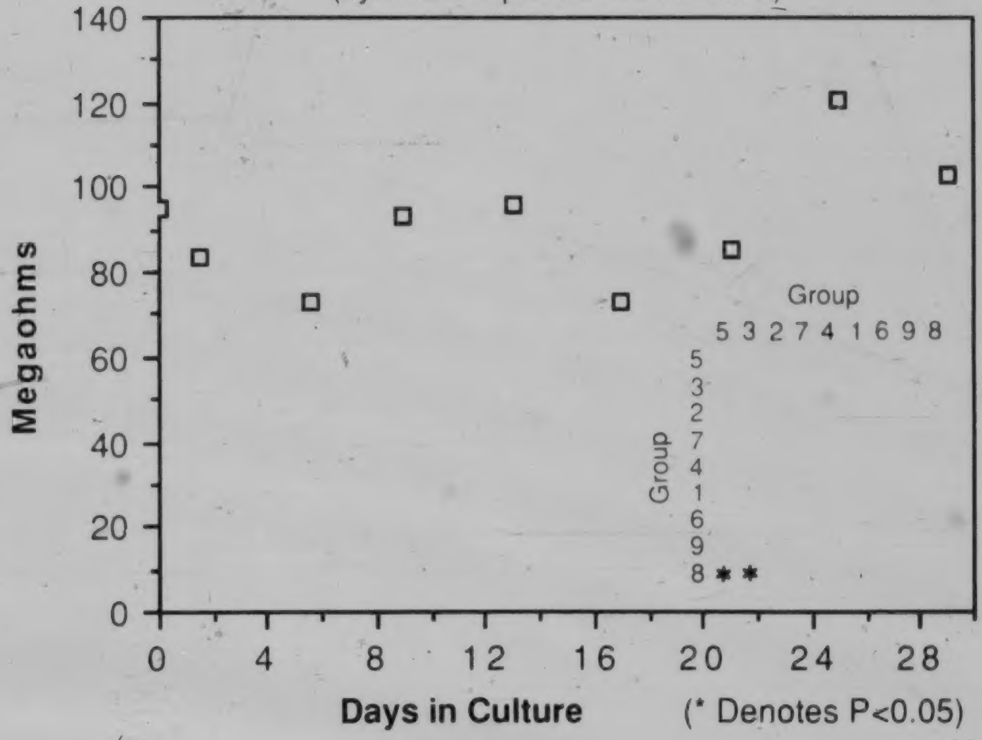


Fig. 14 **Resting Membrane Potential**

(symbols represent n>30 cells)

Fig. 15 **Input Resistance**

(symbols represent n>30 cells)

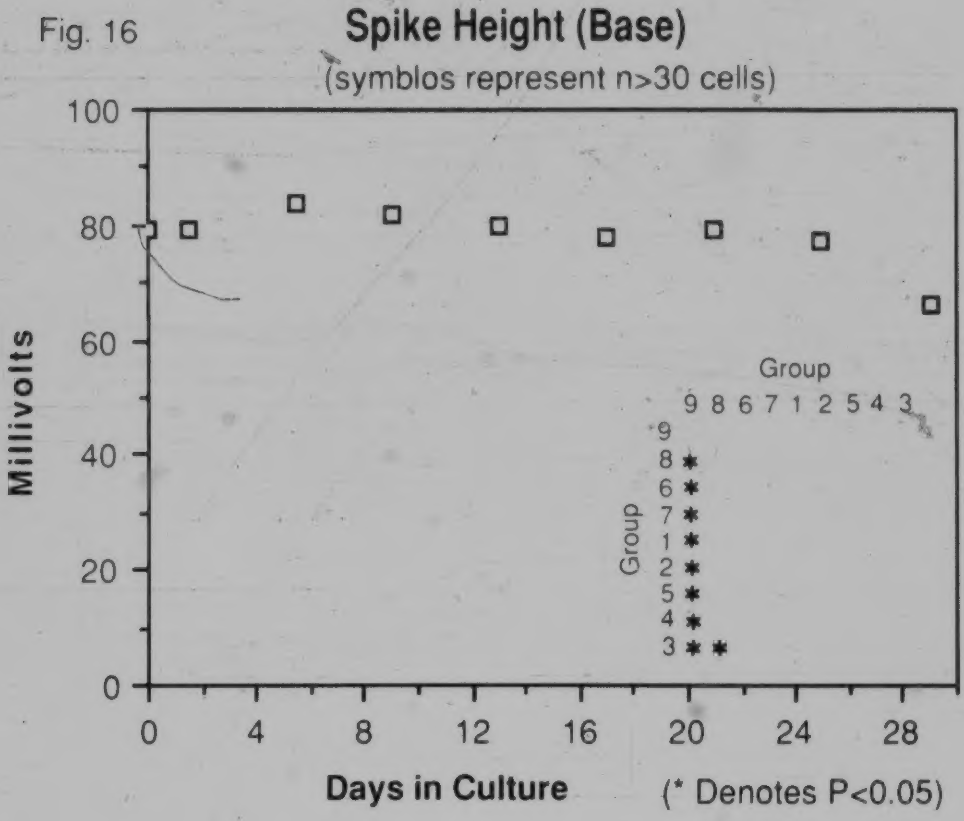


(* Denotes $P<0.05$)

II. CAN THE ELECTROPHYSIOLOGICAL CHANGES OBSERVED IN CULTURE BE REVERSED BY NERVE GROWTH FACTOR?

Nerve Growth Factor (NGF) and other unknown factors may provide trophic support for adult sympathetic, sensory and motor neurones, (Levi-Montalcini, 1976; Gallego et al., 1978; Gordon, 1983). Thus it can be suggested that the electrophysiological changes observed after axotomy and target removal could be part of the cell body response to the lack of trophic support from the periphery. Therefore, if the introduction of exogenous applied NGF into the standard culture medium of explanted BFSG prevents the electrophysiological effects of axotomy and target deprivation in culture this would support the hypothesis that the alterations in the membrane properties of these ganglionic neurones may be due to a loss of the retrograde transport of NGF from peripheral targets (Korsching & Thoenan 1985). It was found that whilst changes in AHP duration and amplitude could be attenuated by NGF, the trophic factor further increased spike width (Fig. 8).

Fig. 16



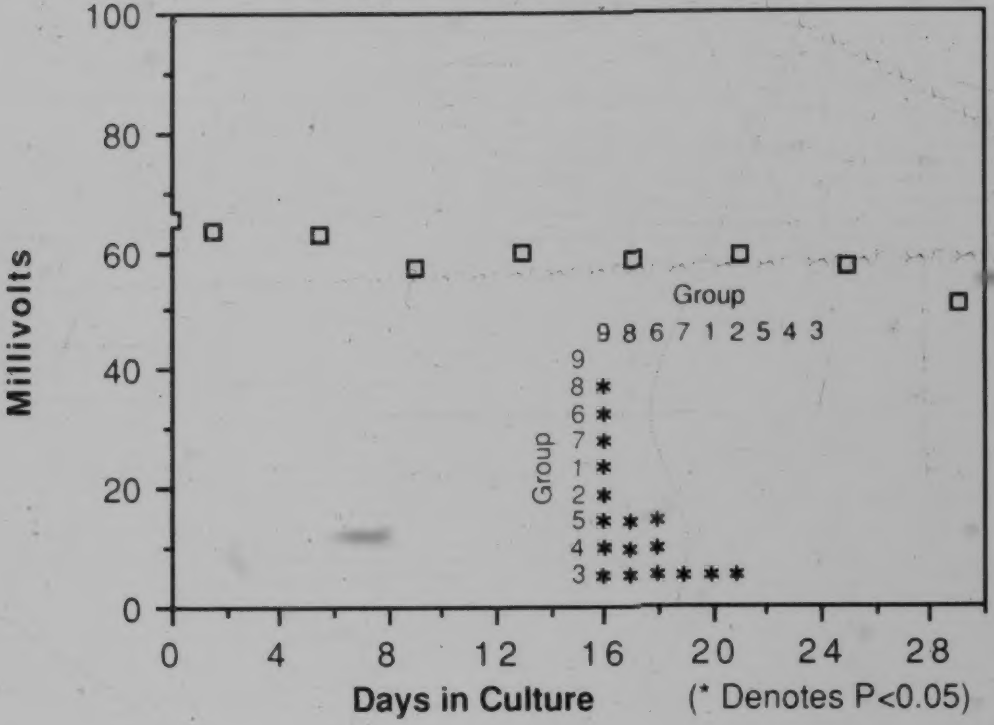
Spike Height (Base)

(symblos represent n>30 cells)

(* Denotes P<0.05)

Fig. 17 **Spike Height (Inflection Point)**

(symbols represent n>30 cells)



A. Effects of NGF on the Electrophysiological Characterisitics of Explanted BFSG Neurones.

The effects of NGF on BFSG neurones was examined by maintaining explants in the presence of NGF (50 ng/ml) at room temperature for 12 to 14 days. Investigations were conducted on 12-14 day old explants primarily due to the finding that the electrophysiological effects of axotomy were maximized after this time period in axotomized BFSG preparations. Use of Duncan's multiple range test enabled simultaneous comparisions to be made between control neurones, neurones studied after 12-14 days in culture, and neurones studied after 12-14 days in culture + NGF.

1. Effects on AHP Parameters:

AHP Duration at 100% Repolarization: After 12-14 days in culture, the AHP duration measured at 100% repolarization was significantly reduced from the mean control value of 290.8 ms to a mean value of 164.0 ms. The presence of NGF in the culture medium prevented the decrease in AHP duration. Figures 8A and 18, show that the mean value for AHP duration in the presence of NGF was statistically indistinguishable from control.

AHP Duration at 75% Repolarization: When the control and experimental groups were compared with respect to AHP duration at 75% repolarization, NGF did not prevent the decrease which occured in explanted ganglia. Furthermore, the 12-14 day group did not differ significantly from the 12-14 day + NGF group, although both groups differed significantly from the control group (Figs. 8A and 19).

Fig.18

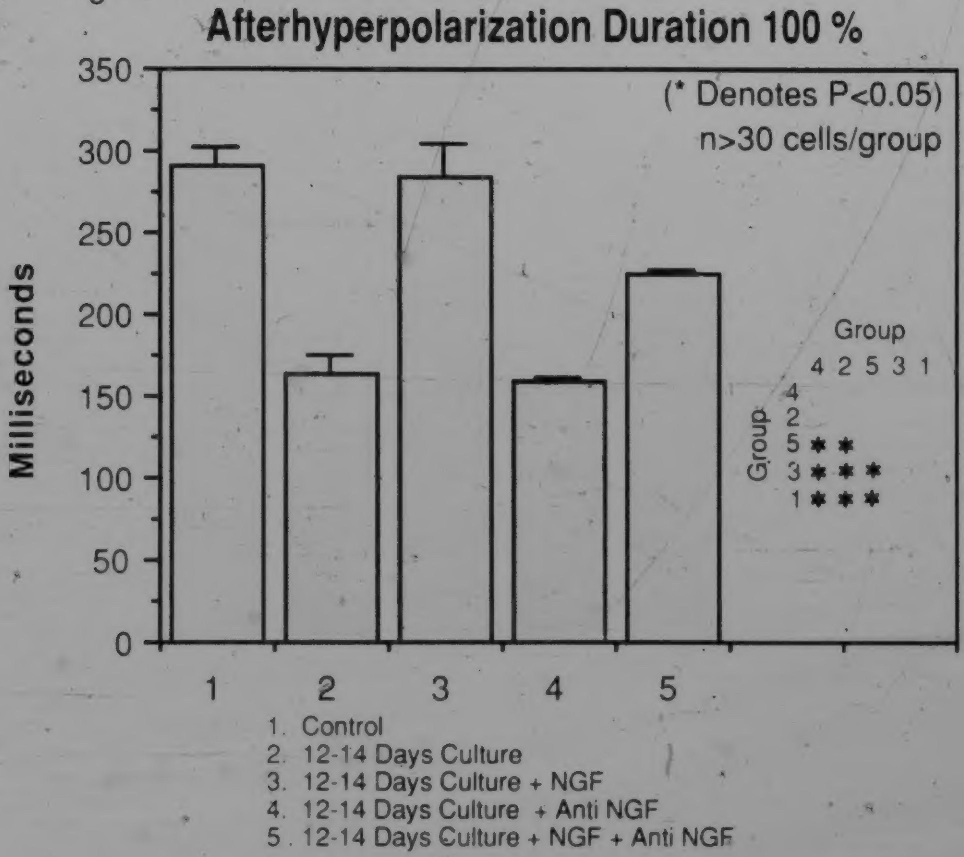
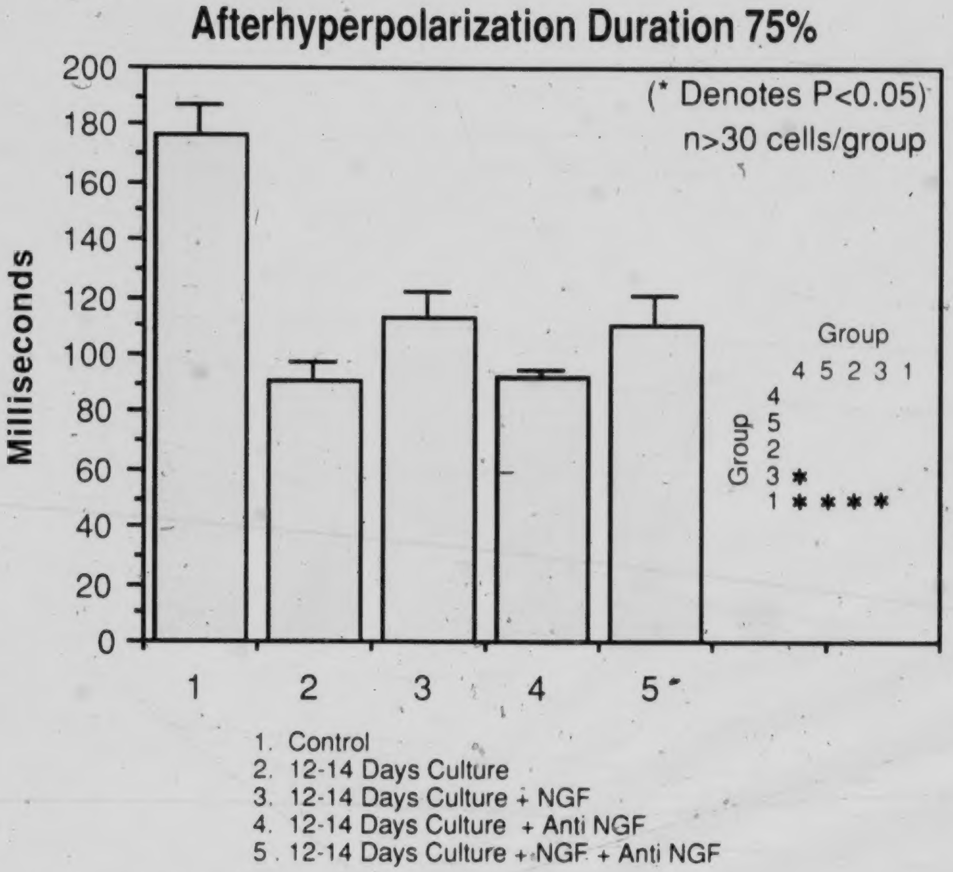

Afterhyperpolarization Duration 100 %
(* Denotes P<0.05)
n>30 cells/group
Milliseconds
0
50
100
150
200
250
300
350
1
2
3
4
5
Group
4 2 5 3 1
Group
4
2
5 * *
3 * * *
1 * * *
1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5 . 12-14 Days Culture + NGF + Anti NGF

Fig. 19


Afterhyperpolarization Duration 75%
(* Denotes P<0.05)
n>30 cells/group
Milliseconds
200
180
160
140
120
100
80
60
40
20
0
1
2
3
4
5
Group
4 5 2 3 1
Group
4
5
2
3 *
1 * * * *
1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5. 12-14 Days Culture + NGF + Anti NGF

AHP Amplitude: As previously shown, the AHP amplitude of APs recorded from neurones after 12-14 days in culture was significantly less than that of control neurones. Although, recovery of AHP amplitude to control values was not complete after 12-14 days in the presence of NGF (Figs. 8A and 20), the reduction in AHP amplitude was not as pronounced as that produced in the absence of NGF.

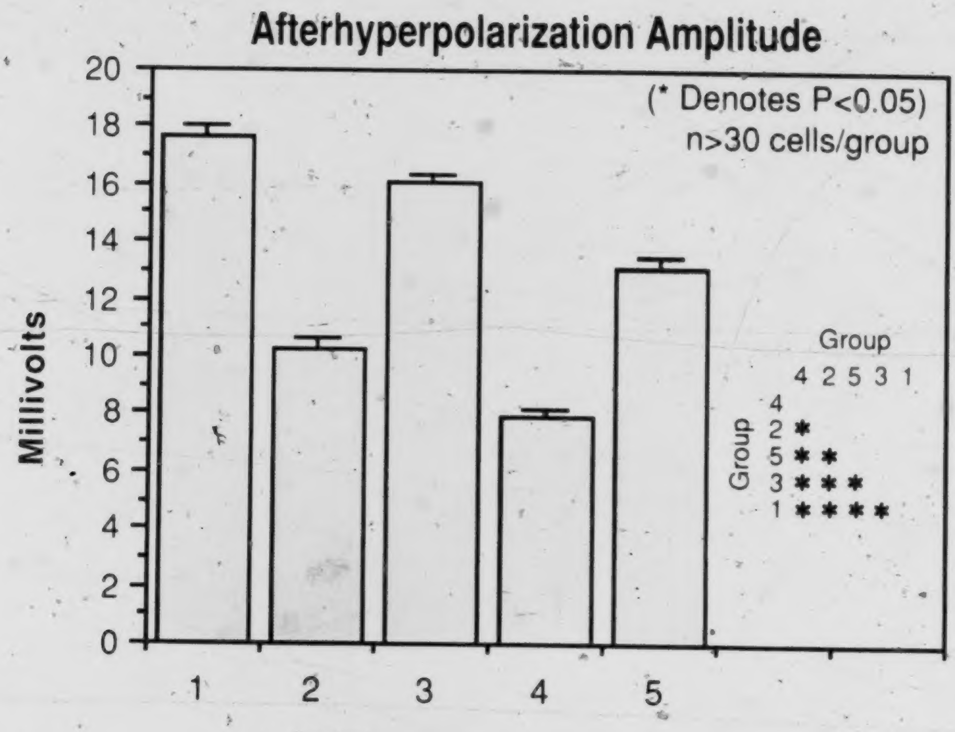
2. Effects on Spike Duration:

As already mentioned, a significant and progressive increase in spike width occurs as BFSG neurones are maintained in explant culture. This effect was not prevented by NGF. On the contrary, inclusion of trophic factor in the medium for 12-14 days resulted in a further increase in spike width (Figs. 8B and 21).

3. Effects on Rheobase:

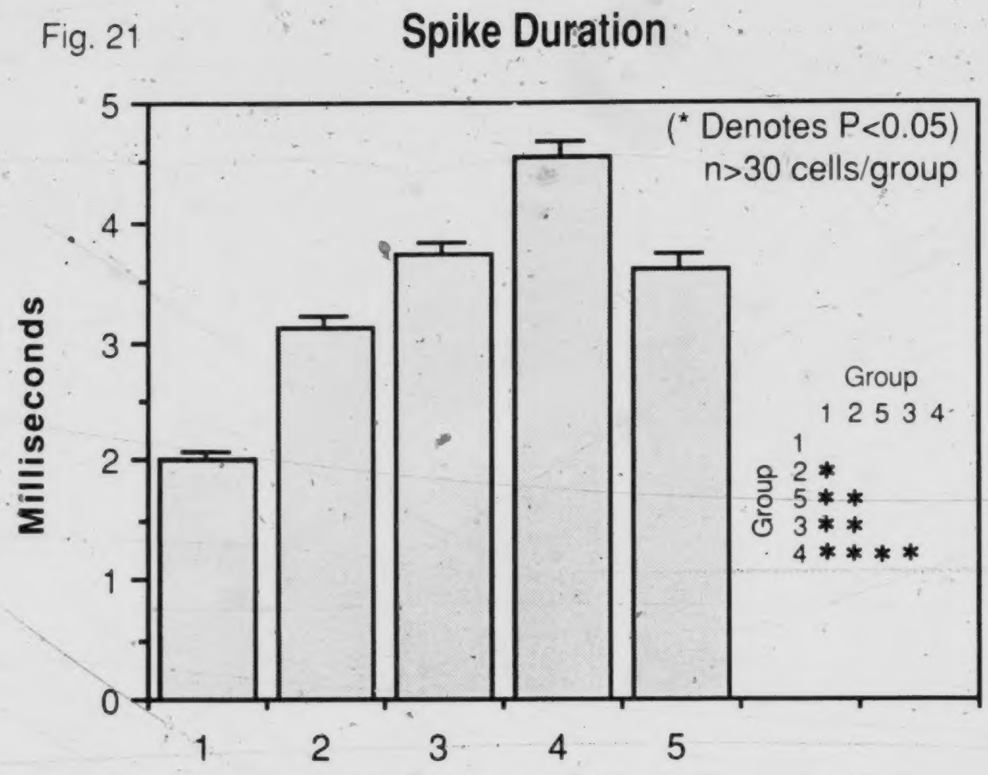
Rheobase was also markedly affected by culture duration and NGF duration and NGF addition. Explant cultures, 12-14 days of age had a mean rheobase value of 0.324 nA, which was significantly greater than the mean rheobase value of 0.172 nA for the control condition. NGF attenuated this change; in the presence of NGF, 12-14 day old cultures exhibited a mean rheobase value of 0.228 nA. Figure 22 shows that the rheobase of cells in the presence of NGF for 12-14 days was statistically indistinguishable from control.

Fig. 20



1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5. 12-14 Days Culture + NGF + Anti NGF

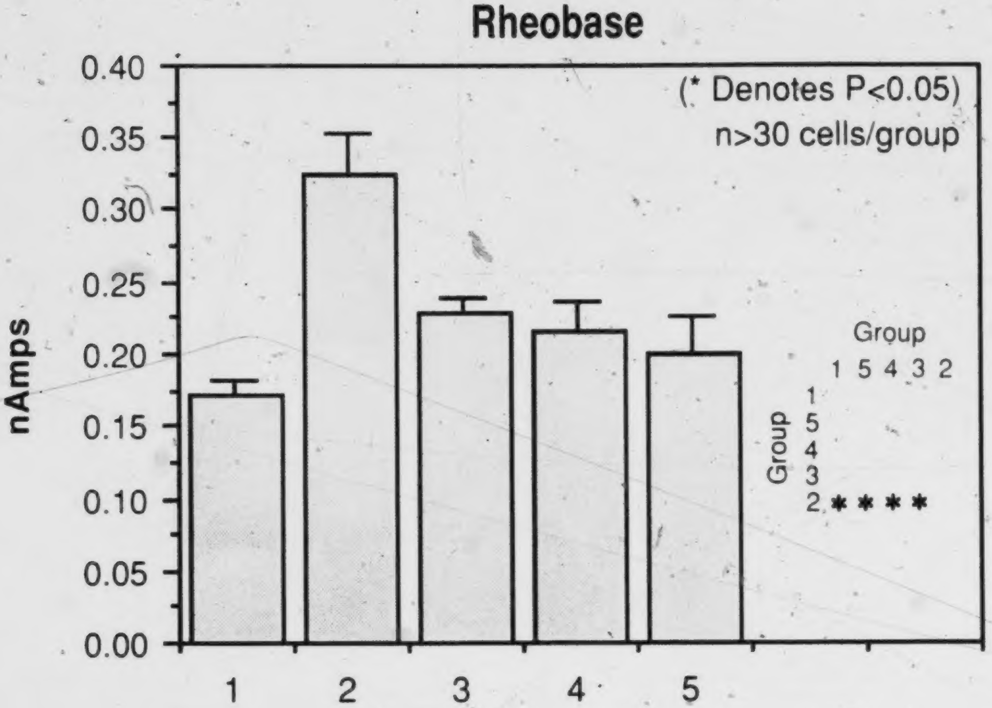
Fig. 21



Spike Duration

1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5. 12-14 Days Culture + NGF + Anti NGF

Fig. 22



1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5. 12-14 Days Culture + NGF + Anti NGF

4. Effects on Other Parameters.

Resting membrane potential (RMP): No significant differences were observed between the RMPs of the control, 12-14 day and 12-14 day + NGF conditions, (Fig. 23)

Input Resistance: Figure 24 shows that no significant differences in input resistance exist between the control and either experimental group (12-14 days and 12-14 days culture + NGF) or between the experimental groups themselves.

Spike Height: The addition of NGF to the culture medium for a 12-14 day period significantly decreased spike height when measured at both the base and at the inflection point (Figs. 24 and 25).

III. ARE THE ELCTROPHYSIOLOGICAL CHANGES SEEN IN CULTURE DUE TO THE LOSS OF ENDOGENOUS NGF ?

The finding that application of NGF to explant cultures of BFSG prevents many of the electrophysiological changes produced by axotomy and target removal is consistent with the hypothesis that NGF may be a trophic factor responsible for the maintenance of the normal electrophysiological properties of the paravertebral sympathetic ganglia neurons in the bullgrog.

Fig. 23

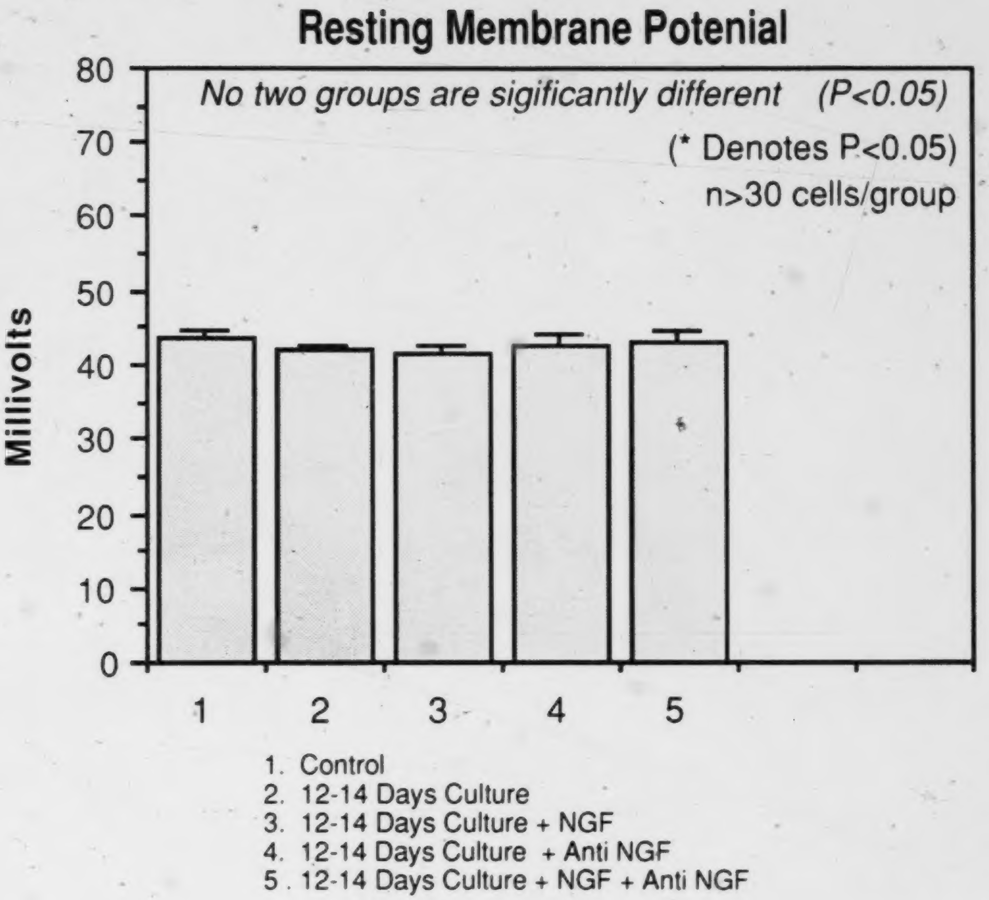
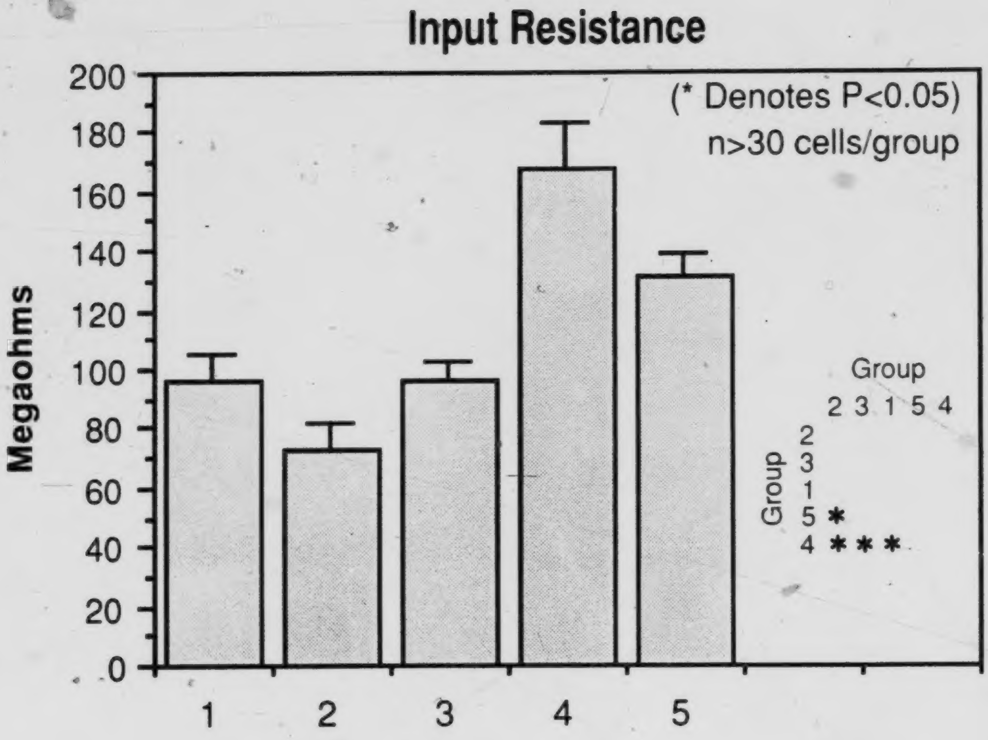

Resting Membrane Potenial
No two groups are sigificantly different (P<0.05)
(* Denotes P<0.05)
n>30 cells/group
Millivolts
80
70
60
50
40
30
20
10
0
1
2
3
4
5
1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5 . 12-14 Days Culture + NGF + Anti NGF

Fig. 24



1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5. 12-14 Days Culture + NGF + Anti NGF

Fig. 25

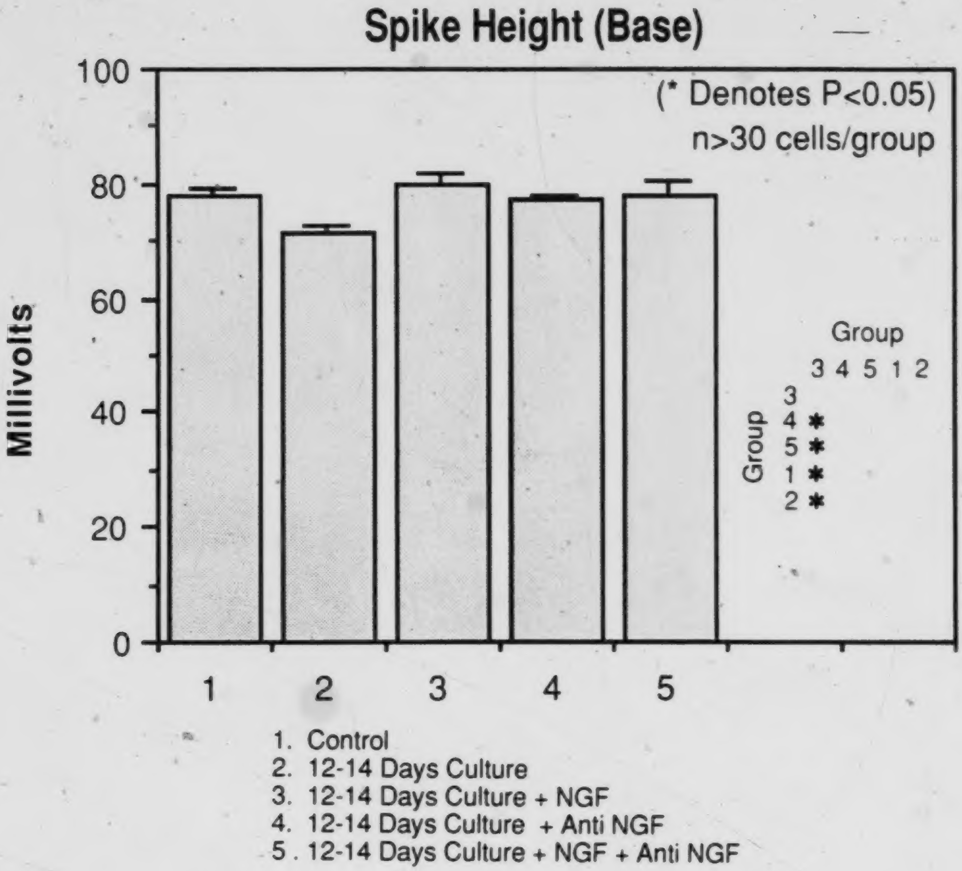
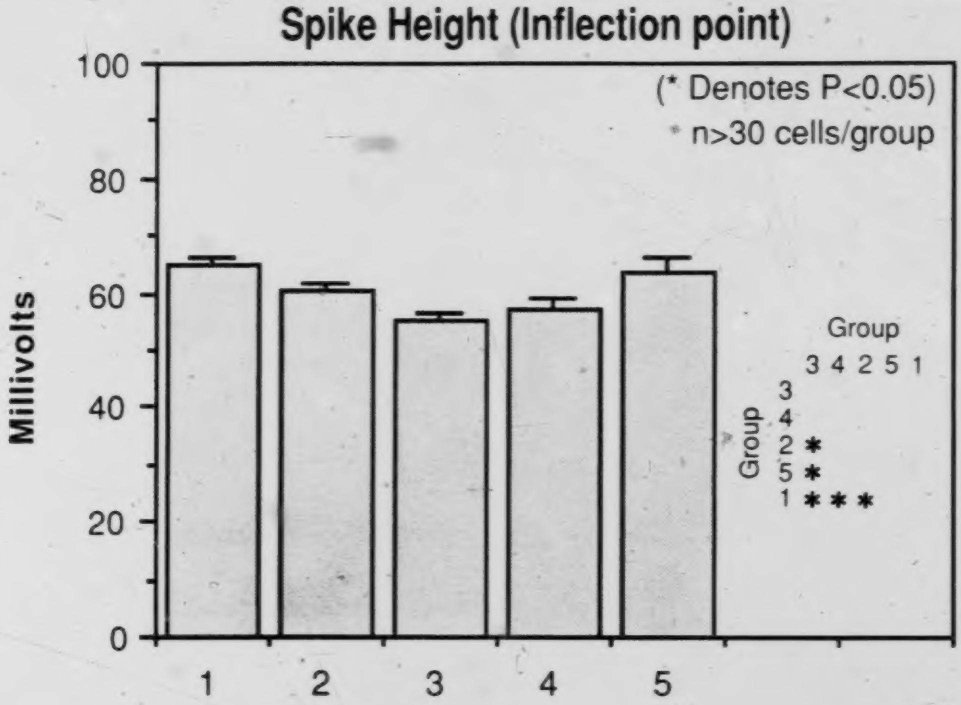

Spike Height (Base)
(* Denotes P<0.05)
n>30 cells/group
Millivolts
100
80
60
40
20
0
1
2
3
4
5
Group
3 4 5 1 2
Group
3
4 *
5 *
1 *
2 *
1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5 . 12-14 Days Culture + NGF + Anti NGF

Fig. 26



1. Control
2. 12-14 Days Culture
3. 12-14 Days Culture + NGF
4. 12-14 Days Culture + Anti NGF
5. 12-14 Days Culture + NGF + Anti NGF

Nevertheless, exogenously applied NGF initiates growth and/or regeneration in several types of nerve cells. (Levi-Montalcini, 1976; Levi-Montalcini et al., 1985; Chalazontis et al.,1987; Hatanaka et al., 1987). Therefore, with respect to BFSG neurones, the apparent reversal of the electrophysiological effects of axotomy and culture duration by NGF may be a " pharmacological" effect, i.e. NGF may not be the trophic substance responsible for maintaining the normal electrophysiological properties of these neurones, but may simply act as an adequate substitute for the true trophic substance.

To test this possibility, any endogenous NGF to which the neurones of the explant might have access to was neutralized. Glial tissue is a source of endogenous NGF (Assouline et al.,1978; Abrahamson et al.,1986) and NGF receptors on glial tissue increase in number after axotomy. Thus, explanted BFSG neurones may have access to a certain unknown amount of endogenous, amphibian NGF. This endogenous NGF could perhaps be neutralized by the addition of NGF antibodies (anti-NGF) to the culture medium. Therefore, the electrophysiological changes produced by axotomy and target removal should be enhanced by the addition of anti-NGF. If this occurs, it provides further evidence supporting the hypothesis that NGF is a trophic substance that is involved in the regulation of the electrophysiological properties of BFSG neurones.

A. Effects of NGF antibodies on the Electrophysiological Characteristics of Explant Cultures of Bullfrog Sympathetic Ganglia.

Explanted BFSG neurones were incubated with anti-NGF (affinity isolated, IgG, 0.5 ug/ml), at room temperature for culture durations of 12-14 days. The parameters of these neurones maintained in the presence of the antibody were measured, and simultaneous comparisons were made between these results and the control, 12-14 days culture duration and 12-14 days culture duration + NGF groups using Duncan's multiple range test. In general, anti-NGF enhanced the changes seen in spike width and AHP amplitude but not AHP duration.

1. Effects on AHP Parameters:

AHP Duration at 100% and 75% Repolarization: With respect to AHP duration measured at both 75 and 100% repolarization, no significant differencewas found to exist between the AHP durations of APs recorded from explants maintained in standard medium alone, and those maintained in standard medium plus anti-NGF, (Figs. 18 and 19).

Effects on AHP Amplitude: The maintenance of BFSG explants in culture medium containing anti-NGF for periods of 12-14 days, significantly reduced the AHP amplitude of the APs generated by these neurones, to values lower than any of the previous conditions (Figs. 8A and 20). Most importantly, anti-NGF significantly reduced the AHP amplitude below values of AHP amplitude measured from the APs produced by neurones in explants maintained for 12-14 days in standard culture medium alone.

2. Effects on Spike Duration:

Addition of anti-NGF significantly enhanced spike duration (Figs. 8B and 21), to values greater than that produced by any other experimental condition. Therefore anti-NGF enhances the effects of target deprivation and culture duration with respect to this parameter.

3. Effects on Rheobase:

Figure 22 shows that anti-NGF does not enhance the increase in rheobase normally seen when BFSG are maintained in culture for 12 - 14 days. Indeed, the antibodies seem to have a NGF-like effect in that they seem to prevent the effect of target deprivation on rheobase.

4. Effects on Other Parameters.

Resting membrane potential (RMP): RMP was not affected by any of the culture conditions so far discussed (Fig. 23).

Input Resistance: The presence of anti-NGF significantly increases the input resistance of explanted BFSG neurones. The calculated value of input resistance for those explants maintained in culture medium with anti-NGF is twice the value of input resistance calculated for cultures of the same age maintained in standard medium alone (Fig. 24).

Spike Height (base): Spike height measured from the base of the AP was not markedly affected by anti-NGF addition. No significant differences existed between control, 12-14 days in standard medium and 12-14 days in standard medium + anti-NGF. (Fig. 25)

Spike height (inflection point): With respect to spike height measured at the inflection point, anti-NGF significantly reduced amplitude to a level below that of the control group, but no difference existed between the spike height for this group and values for 12-14 day old cultures in standard culture medium (Fig. 26).

IV. PROOF OF THE EFFECTIVENESS OF NGF ANTIBODIES.

The results of some of the above experiments are consistent with the hypothesis that the alterations of the membrane properties of BFSG neurones induced by anti-NGF may be due to the neutralization of endogenous NGF within the explant. To test whether anti-NGF was successfully introduced into the culture medium and that it was not lost during the sterilization of the medium, (refer to methods) and that it was effective in neutralizing NGF, mouse salivary gland NGF and an excess of anti-NGF were simultaneously introduced into the culture medium. If the anti-NGF was successfully introduced and remained effective, it should decrease the effectiveness of the exogenously applied mouse NGF on the electrophysiological properties of the explanted BFSG neurones.

A. Effects of the presence of NGF and NGF antibodies on the electrophysiological properties of explanted bullfrog sympathetic ganglia.

Since lug of anti-NGF normally neutralizes lug of NGF (R. Murphy personal communication), concentrations of 0.5 ug/ml of anti-NGF and 50ng/ml of NGF were utilized in culture medium bathing explanted ganglia for culture durations of 12-14 days. Therefore, an excess of anti-NGF was present _in vitro_. The electrophysiological properties of these neurones were examined and simultaneous comparisons were made between the values of the AP parametres recorded from neurones under these conditions and the control, 12-14 days culture duration, 12-14 days + NGF and 12-14 days + anti-NGF groups, using Duncan's multiple range test. In general, the presence of NGF antibodies attenuated the response to NGF.

1. Effects on AHP Parameters.

AHP Duration at 100% Repolarization: In the presence of NGF plus anti-NGF, AHP duration measured at 100% repolarization was significantly less than that measured in the NGF alone condition. Nevertheless, it was significantly greater than that measured in the presence of NGF- antibodies (Fig. 18).

AHP Duration at 75% Repolarization: NGF and anti-NGF applied simultaneously to the culture medium reduced the value of AHP duration measured at 75% repolarization, when compared to the mean value obtained in explants maintained in NGF alone. This difference was not stastically significant. Also, no significant difference existed between the NGF and anti-NGF simultaneous application and the anti-NGF alone condition (Fig. 19)

AHP Amplitude: The AHP amplitude in the presence of NGF plus anti NGF was reduced to a value significantly less that that measured in the presence of NGF alone. Further comparisons demonstrated that the value of AHP amplitude recorded from neurones in explants maintained in medium containing anti-NGF alone was significantly less than the value of AHP amplitude recorded from explants maintained in NGF plus anti NGF, (Fig. 20).

2.Effects on Spike Duration.

The A.P. durations measured from APs generated by cells in explants exposed to NGF plus anti-NGF, did not differ significantly from the AP durations of those cells exposed only to NGF. Neverthless, when a comparision was made between cells exposed to NGF plus anti-NGF, and those cells maintained in the presence of anti-NGF alone, the AP duration of cells exposed only to anti-NGF was significantly greater (Fig. .21).

3.Effects on Rheobase.

The addition of NGF plus anti-NGF to explants effectively reduces the threshold for AP generation to that of control cells. No significant differences were found to exist between this condition and the NGF or anti-NGF alone conditions (Fig. .22)

4.Effects on Other Parameters

Resting membrane potential (RMP): Figure 23 shows that changing culture conditions did not affect the value of RMP for any of the cells within the explant cultures, and no significant differences existed between any of the groups compared.

Input Resistance: There were no significant differences in input resistance found between the NGF plus anti-NGF condition, and the NGF alone or anti-NGF alone conditions, (Fig. 24). Although NGF plus anti-NGF simultaneously introduced into the culture medium increased input resistance to a value greater than the NGF alone condition, it did not increase input resistance to that observed in the presence of anti-NGF alone.

Spike Height (base and inflection point): Spike height measured from the base of the AP was increased by NGF application, and this increase was reduced in the presence of NGF plus anti-NGF. The reduction in spike height (measured from the inflection point) produced by NGF was not produced when NGF plus anti-NGF was included in the medium (Fig. 26).

V. AN INVESTIGATION OF THE TIME COURSE OF THE EFFECTS OF NGF.

Since NGF can induce extensive neurite outgrowth within 24 h. (Partlon et al., 1971), and protein synthesis (Acheson et al.,1987) and cyclic nucleotide synthesis within minutes of application to cells (Ikeno et al., 1979), the question arises as to the rate at which NGF mediates its effects on the electrophysiological characteristics of explanted BFSG neurones. To start to address this question , experiments were carried out to determine if the effects of NGF could be observed within a relatively short period of time.

A. Effect of a 24h NGF Exposure on the Electrophysiological Properties of Explanted BFSG Neurones.

Comparisons were made between 12-14 day old explant cultures maintained in standard medium and 12-14 day old explant cultures exposed to NGF (50ng/ml) for the final 24h of the culture periods. Statistical differences were assessed using the Student's two-tailed unpaired, t-test for continuous variables.

1. Effect on AHP Parameters:

AHP Duration at 100% and 75% Repolarization : Application of NGF for 24h significantly enhanced AHP duration measured at 100% (Fig. 27A) but not at 75% (Fig. 27b).

AHP Amplitude: AHP amplitude also responded to the 24hr NGF exposure. AHP amplitude was significantly increased from 10.3 mV in the absence of NGF to 13.7 mV after the 24h NGF treatment, (Fig. 27c).

2.Effect on Spike Duration.

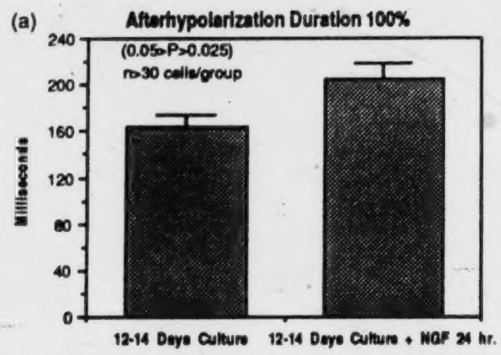
The NGF treatment did not effectively enhance or reduce spike duration. Therefore no significant difference was found to exist between the 12-14 day old explants in standard culture medium and same age cultures treated for 24hrs with NGF (Fig. 27d).

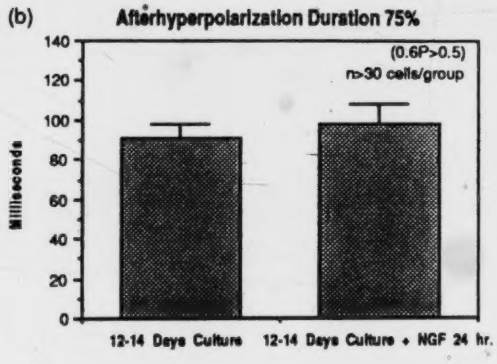
3.Effect on Rheobase.

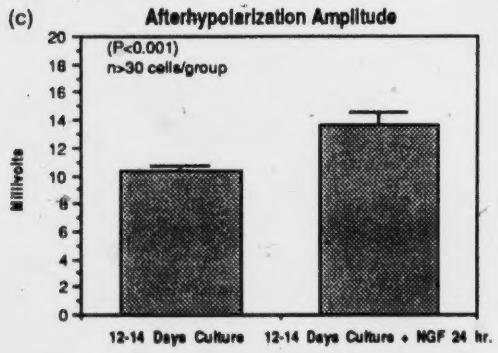
As a consequence of NGF addition, the current required for AP generation was markedly reduced towards that required in acute control sympathetic neurones. Thus a significant difference existed between calculated rheobase values for the 12-14 day explants in standard medium and those exposed to NGF for 24 hours (Fig. 27e).

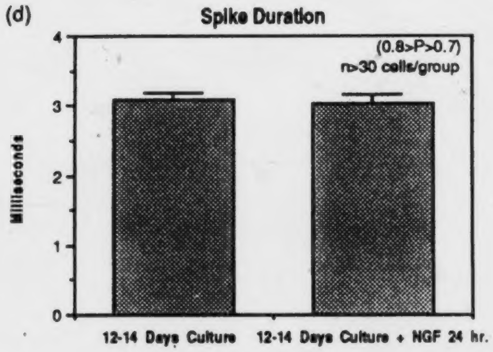
FIGURE 27 :

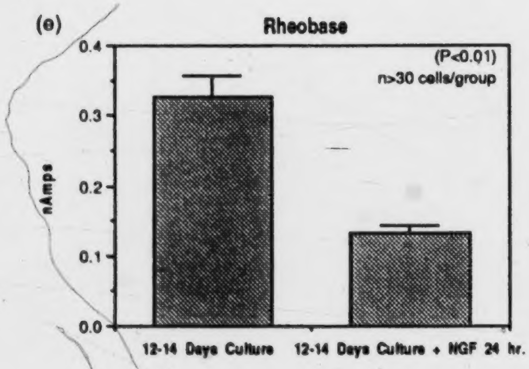
Effect of a 24h NGF Exposure on the Electrophysiological Properties of Explanted BFSG Neurones.


(a) Afterhypolarization Duration 100%


(b) Afterhyperpolarization Duration 75%


(c) Afterhypolarization Amplitude


(d) Spike Duration


(e) Rheobase

4. Effect on Other Parameters.

The RMP, input resistance and spike heights measured from both the base and inflection point of the AP were found not to differ significantly when a comparison between 12-14 day cultures with and without NGF was performed (Fig. 28).

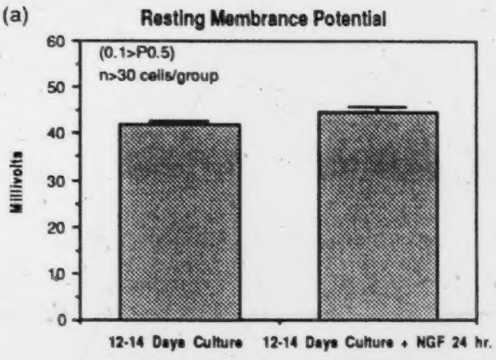
VI. DOES THE LENGTH OF THE SEVERED AXON INFLUENCE THE ELECTROPHYSIOLOGICAL EFFECTS OF AXOTOMY AND TARGET REMOVAL?

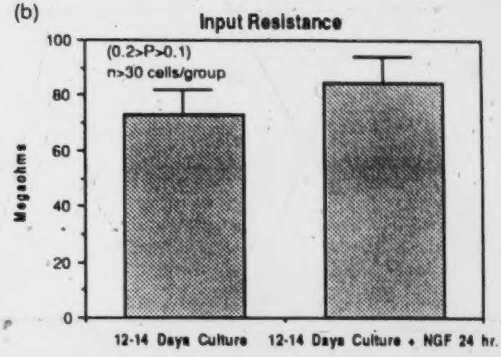
Since the point of axon section in explanted BFSG neurones was at the point where the rami communicantes emerge from the ganglion, the entire length of the axon contained in the associated spinal nerve was removed. NGF is known to be retrogradedly transported by axons of several neuronal types (Hendry et al., 1974; Korsching et al., 1983), and has also been shown to be produced by glial tissue (Taniuchi et al.,1988; Abrahanson et al.,1986; Assouline et al., 1987). Therefore, if BFSG neurones were explanted with the spinal nerve remaining attached to the ganglion but cut at a point distant from the ganglion body, any NGF produced by the surrounding glial tissue or any NGF remaining in the axon immediately after axotomy, may delay the onset of the axotomy induced electrophysiological changes. As a consequence, the effects of axotomy would be

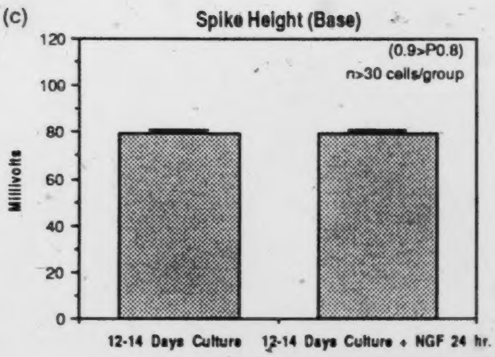
FIGURE 28:

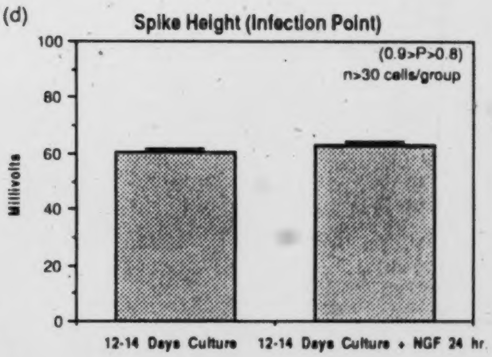
Effect of a 24h NGF Exposure on the Electrophysiological Properties of Explanted BFSG Neurones.

(OTHER PARAMETERS).


(a)
Resting Membrance Potential
(0.1>P0.5)
n>30 cells/group
Millivolts
60
50
40
30
20
10
0
12-14 Days Culture
12-14 Days Culture + NGF 24 hr.


(b)
Input Resistance
(0.2>P>0.1)
n>30 cells/group
Megaohms
100
80
60
40
20
0
12-14 Days Culture
12-14 Days Culture + NGF 24 hr.


(c)
Spike Height (Base)
(0.9>P0.8)
n>30 cells/group
Millivolts
120
100
80
60
40
20
0
12-14 Days Culture
12-14 Days Culture + NGF 24 hr.


(d)
Spike Height (Infection Point)
(0.9>P>0.8)
n>30 cells/group
Millivolts
100
80
60
40
20
0
12-14 Days Culture
12-14 Days Culture + NGF 24 hr.

less pronounced (Fig. 29a and b and plate 1).

A. Effect of axon length on the electrophysiological properties of explanted bullfrog sympathetic ganglia.

The electrophysiological properties of 12-14 day old explants of BFSG maintained in standard culture medium with a 0.8 cm section of their corresponding spinal nerves remaining attached to the ganglion, were investigated. The AP parameters measured from these cells, were compared to those obtained from 12-14 day old explants maintained in standard culture medium using Student's two-tailed unpaired t-test for continuous variables.

1. Effect on AHP parameters:

AHP Duration at 100% and 75% Repolarization: Although no significant difference in AHP duration measured at both the 100% and 75% levels was found to exist between those explants in which the axon was severed close to the cell body and those in which axons were severed distally in the spinal nerve, the presence of an axon did result in a less severe reduction in AHP duration (Fig. 30a and 30b).

AHP Amplitude: The presence of an elongated severed axon did not prevent the reduction in AHP amplitude. No significant differences were found to exist between 12-14

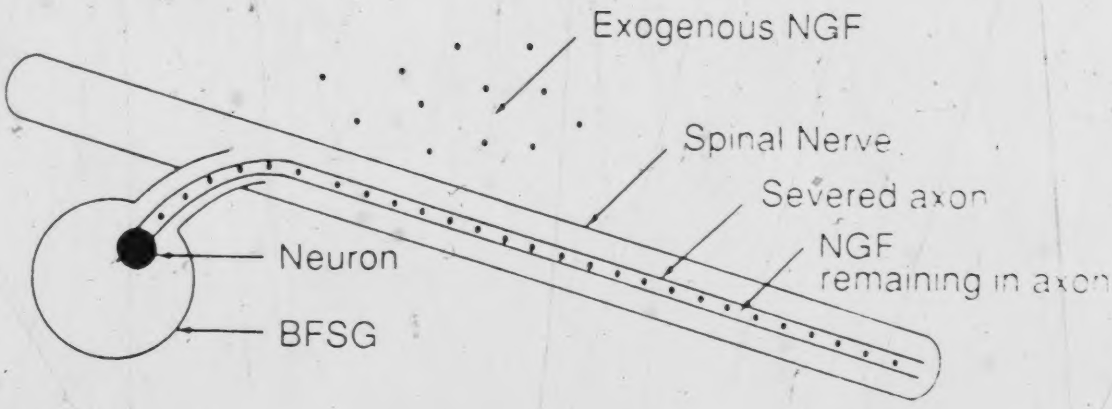
FIGURE 29 : SCHEMATIC DIAGRAMS OF BFSG EXPLANT PREPARATIONS

a) GANGLION WITH ATTACHED SPINAL NERVE, DISPLAYING NGF BEING RETOGRADELY TRANSPORTED DOWN THE AXON TOWARD THE CELL BODY.

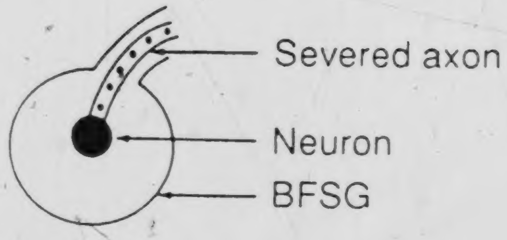
b) GANGLION WITH SPINAL NERVE SEVERED AT THE RAMUS COMMUNICANS. THERE IS LESS RETROGRADE TRANSPORT OF NGF IN THIS TYPE OF PREPARATION.

Fig. 29

A Spinal Nerve Intact


Exogenous NGF
Spinal Nerve
Severed axon
NGF
remaining in axon
Neuron
BFSG

B Spinal Nerve Severed


Severed axon
Neuron
BFSG

THE QUALITY OF THIS MICROFICHE IS HEAVILY DEPENDENT UPON THE QUALITY OF THE THESIS SUBMITTED FOR MICROFILMING.

UNFORTUNATELY THE COLOURED ILLUSTRATIONS OF THIS THESIS CAN ONLY YIELD DIFFERENT TONES OF GREY.

LA QUALITE DE CETTE MICROFICHE DEPEND GRANDEMENT DE LA QUALITE DE LA THESE SOUMISE AU MICROFILMAGE.

MALHEUREUSEMENT, LES DIFFERENTES ILLUSTRATIONS EN COULEURS DE CETTE THESE NE PEUVENT DONNER QUE DES TEINTES DE GRIS.

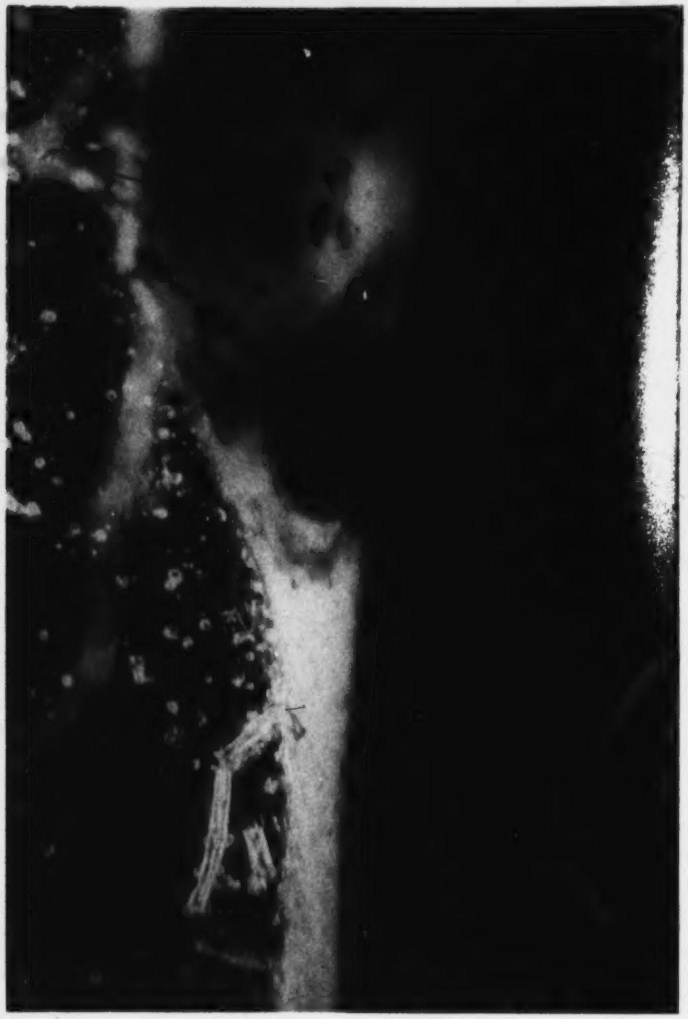


PLATE 1 :
PHOTOMICROGRAPH OF EXPLANTED BFSG ATTACHED BY THE RAMUS COMMUNICANS TO A SPINAL NERVE (MAINTAINED FOR 12 DAYS IN STANDARD MEDIUM).

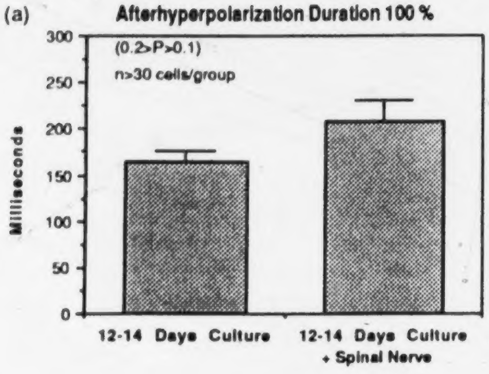
(A) GANGLION

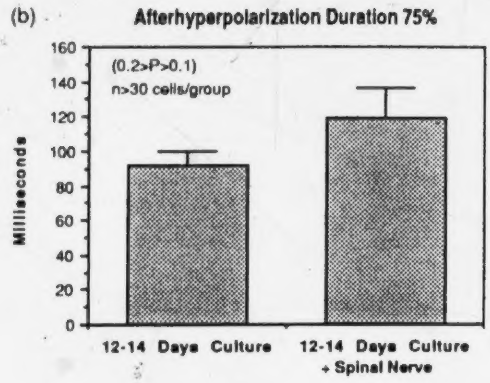
(B) RAMUS COMMUNICANS

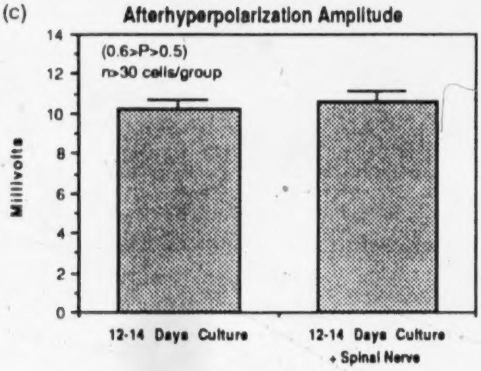
(C) SPINAL NERVE

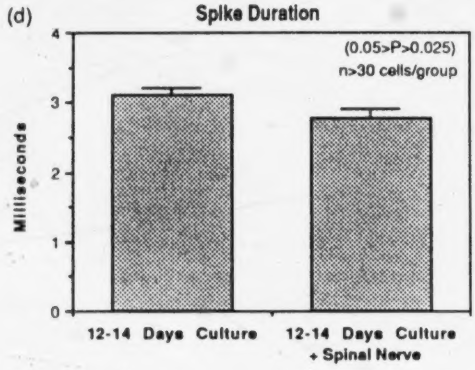
FIGURE 30 :

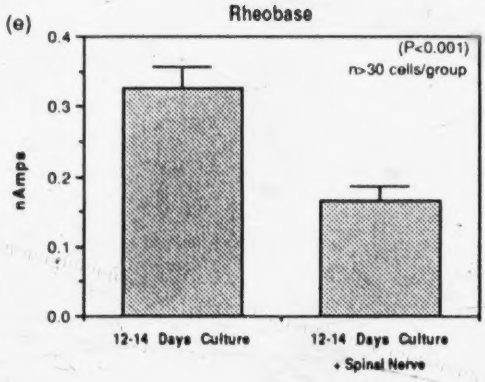
EFFECT OF AXON LENGTH ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF BFSG.


(a)
Afterhyperpolarization Duration 100 %
(0.2>P>0.1)
n>30 cells/group
Milliseconds
300
250
200
150
100
50
0
12-14 Days Culture
12-14 Days Culture + Spinal Nerve


(b)
Afterhyperpolarization Duration 75%
(0.2>P>0.1)
n>30 cells/group
Milliseconds
160
140
120
100
80
60
40
20
0
12-14 Days Culture
12-14 Days Culture + Spinal Nerve


(c)
Afterhyperpolarization Amplitude
(0.6>P>0.5)
n>30 cells/group
Millivolts
14
12
10
8
6
4
2
0
12-14 Days Culture
12-14 Days Culture + Spinal Nerve


(d)
Spike Duration
(0.05>P>0.025)
n>30 cells/group
Milliseconds
4
3
2
1
0
12-14 Days Culture
12-14 Days Culture + Spinal Nerve


(e)
Rheobase
(P<0.001)
n>30 cells/group
nAmps
0.4
0.3
0.2
0.1
0.0
12-14 Days Culture
12-14 Days Culture + Spinal Nerve

day old explants and 12-14 day old explants which attached spinal nerves (Fig. 30c).

2.Effect on Spike Duration:

The duration of the AP recorded from cells in which their axons remained attached was significantly less than that measured from those cells which were axotomized much closer to the cell body. Increased axon length was the nnly experimental condition which attenuated the axotomy induced enhancement of AP duration (Fig. 30d).

3.Effects on Rheobase:

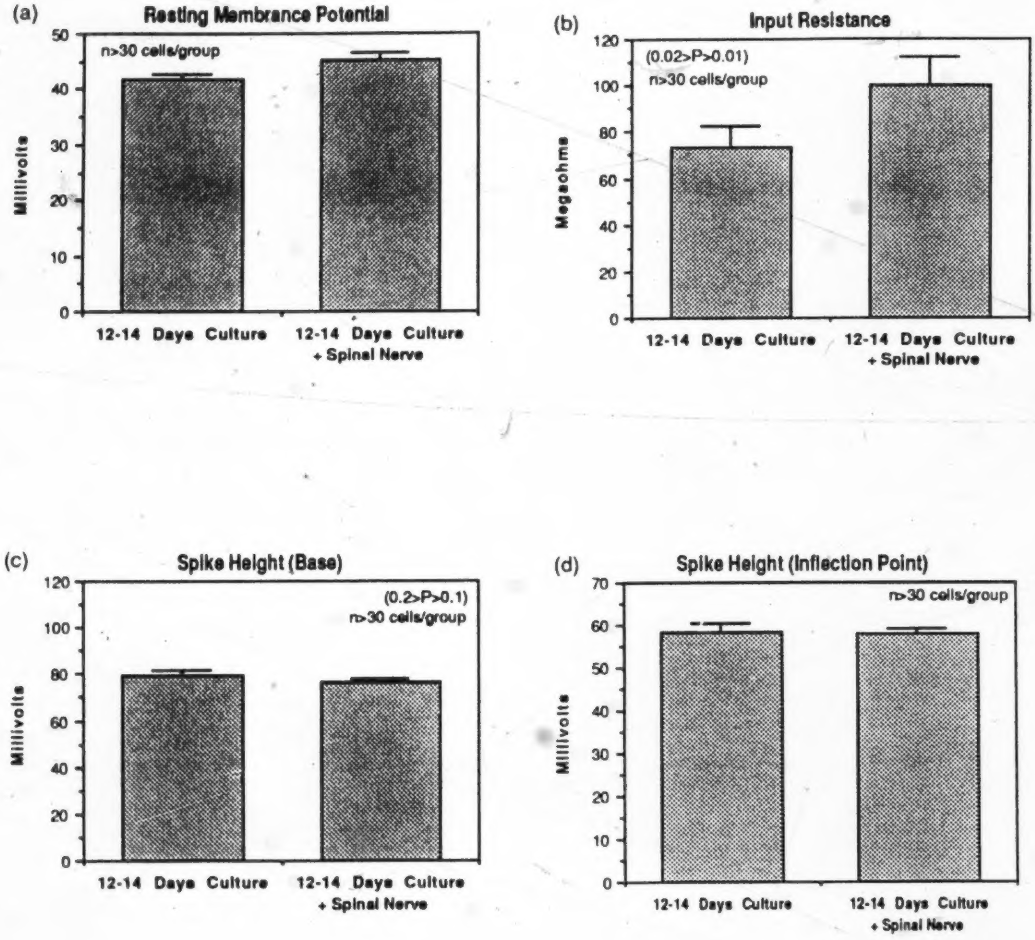
Explants in which spinal nerves remained attached to the ganglion were found to have rheobase valves significantly less than valves calculated for same age explants in which the spinal nerves were removed (Fig. 30e).

4.Effects on Other Parameters:

Resting Membrane Potential (RMP): After 12-14 days in culture the RMP of those explants to which the spinal nerve remained attached was significantly greater than "axotomized" explants (Fig. 31a).

FIGURE 31 :

EFFECT OF AXON LENGTH ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF BFSG (OTHER PARAMETERS).



Input Resistance: Input resistance was significantly increased as a result of the spinal nerve remaining attached to the sympathetic ganglion (Fig. 31b).

Spike Height (Base and Inflection Point): No significant differences were found to exist with respect to spike height when measured from the base or from the inflection point of the AP (Fig. 31c and 31d).

B. Effect of Axon Length and Exogenous Nerve Growth Factor Addition on the Electrophysiological Properties of Explanted Bullfrog Sympathetic Ganglia.

Instead of providing a source of endogenous NGF, the presence of an axon per se could have some separate role in maintaining the normal electrophysiological properties of the cell bodies. This effect could be exerted through several mechanisms, including the involvement of undefined trophic factors. Since either NGF alone, or the presence of an axon alone was able to attenuate, but not completely prevent the electrophysiological changes induced by axotomy, it is possible that the addition of NGF to explant cultures which had axons attached, may allow for complete maintenance of cell body electrophysiological properties. To test this possibility, explants of BFSG with spinal nerves remaining attached to the ganglion, were maintained in standard culture medium plus NGF (50ng/ml) for experimental durations of 12-14 days. The AP parameters measured from these cells were compared to those obtained from 12-14 day old explants maintained in standard culture medium.

1. Effects on AHP Parameters:

AHP Durations at 100% and 75% Repolarization: At both the 100% and 75% measurements of AHP duration, the presence of an elongated axon severed at a point in the spinal nerve and the addition of NGF could not induce AHP durations of similar value to those determined for control cells. Therefore the two conditions remained significantly different (Fig. 32a and 32b).

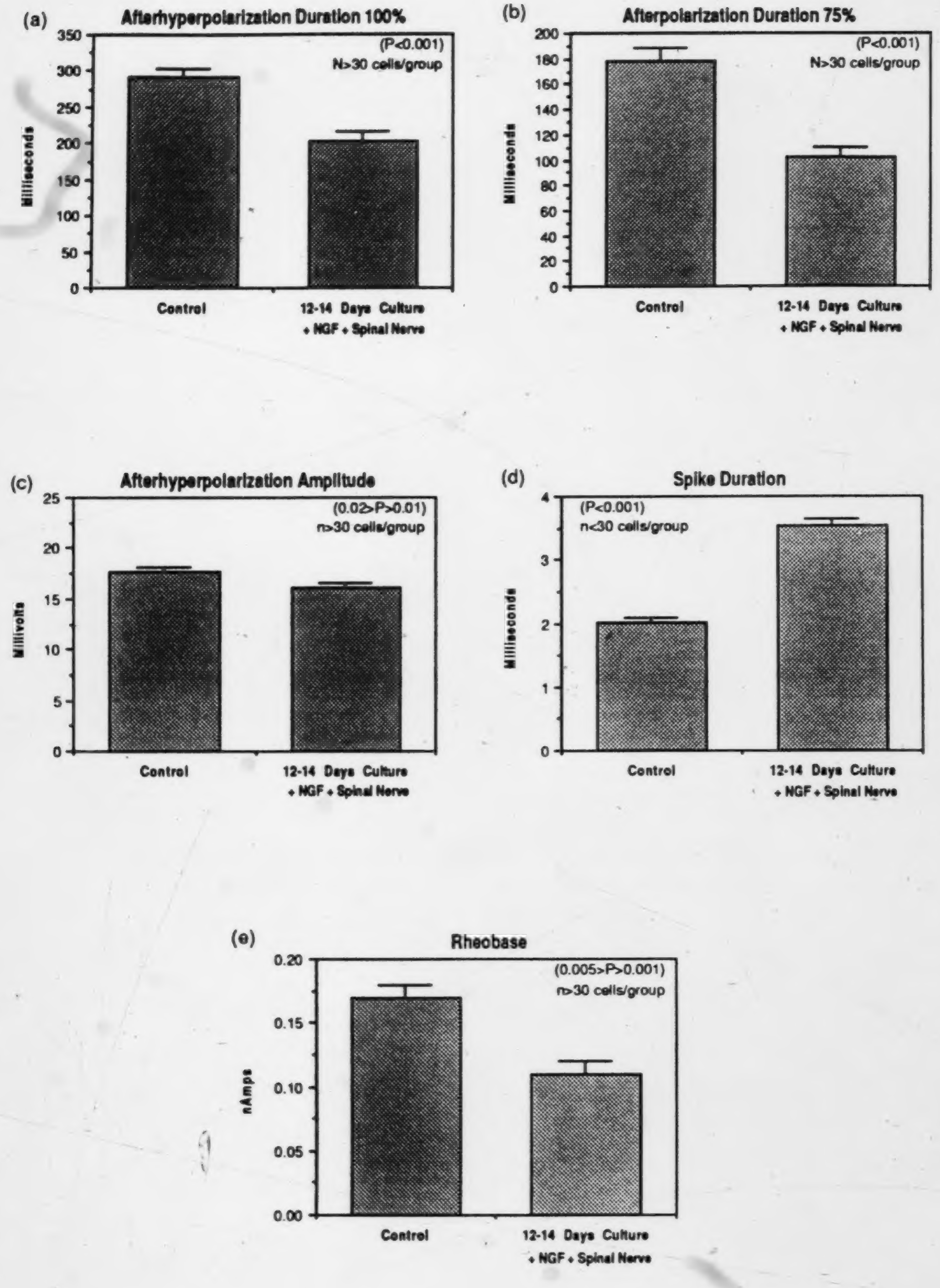
AHP Amplitude: The AHP amplitude measured from explants with spinal nerves maintained for 12-14 days in standard medium plus NGF, was significantly different from the value of AHP amplitude measured for control neurones. It is important to note that this difference was marginal, and NGF addition almost induced a complete recovery of AHP amplitude (Fig. 32c).

2. Spike Duration.

The presence of elongated severed axons and NGF effectively enhanced spike duration in 12-14 day old

FIGURE 32 :

EFFECT OF AXON LENGTH AND EXOGENOUS NERVE GROWTH FACTOR ADDITION ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF EXPLANTED BULLFROG SYMPATHETIC GANGLIA.


(a)
Afterhyperpolarization Duration 100%
(P<0.001)
N>30 cells/group
Milliseconds
350
300
250
200
150
100
50
0
Control
12-14 Days Culture
+ NGF + Spinal Nerve
(b)
Afterpolarization Duration 75%
(P<0.001)
N>30 cells/group
Milliseconds
200
180
160
140
120
100
80
60
40
20
0
Control
12-14 Days Culture
+ NGF + Spinal Nerve
(c)
Afterhyperpolarization Amplitude
(0.02>P>0.01)
n>30 cells/group
Millivolts
25
20
15
10
5
0
Control
12-14 Days Culture
+ NGF + Spinal Nerve
(d)
Spike Duration
(P<0.001)
n<30 cells/group
Milliseconds
4
3
2
1
0
Control
12-14 Days Culture
+ NGF + Spinal Nerve
(e)
Rheobase
(0.005>P>0.001)
n>30 cells/group
nAmps
0.20
0.15
0.10
0.05
0.00
Control
12-14 Days Culture
+ NGF + Spinal Nerve

explants, to a value far greater than that determined for control cells (Fig. 32d). The difference between the two conditions was highly significant and may be attributed to NGF action. This is due to the fact that recordings of spike duration from explants with attached spinal nerves in standard culture medium had spike durations tending towards control values rather than away from control valves as occurs during NGF introduction (Fig. 30d).

3. Effects on Rheobase.

Addition of NGF to medium maintaining explants with attached spinal nerves induced a pronounced reduction in the amount of current required to generate an AP. The value of rheobase for cells maintained under these conditions was less than that required for AP firing in acutely axotomized control cells. The difference between the two conditions is highly significant (Fig. 32d).

4. Effects on other parameters:

No significant difference was found between the control and experimental conditions with respect to input resistance, resting membrane potential and and spike height (base and inflection point) (Fig. 33).

VII. OBSERVATIONS OF THE EFFECTS OF CHANGING CULTURE

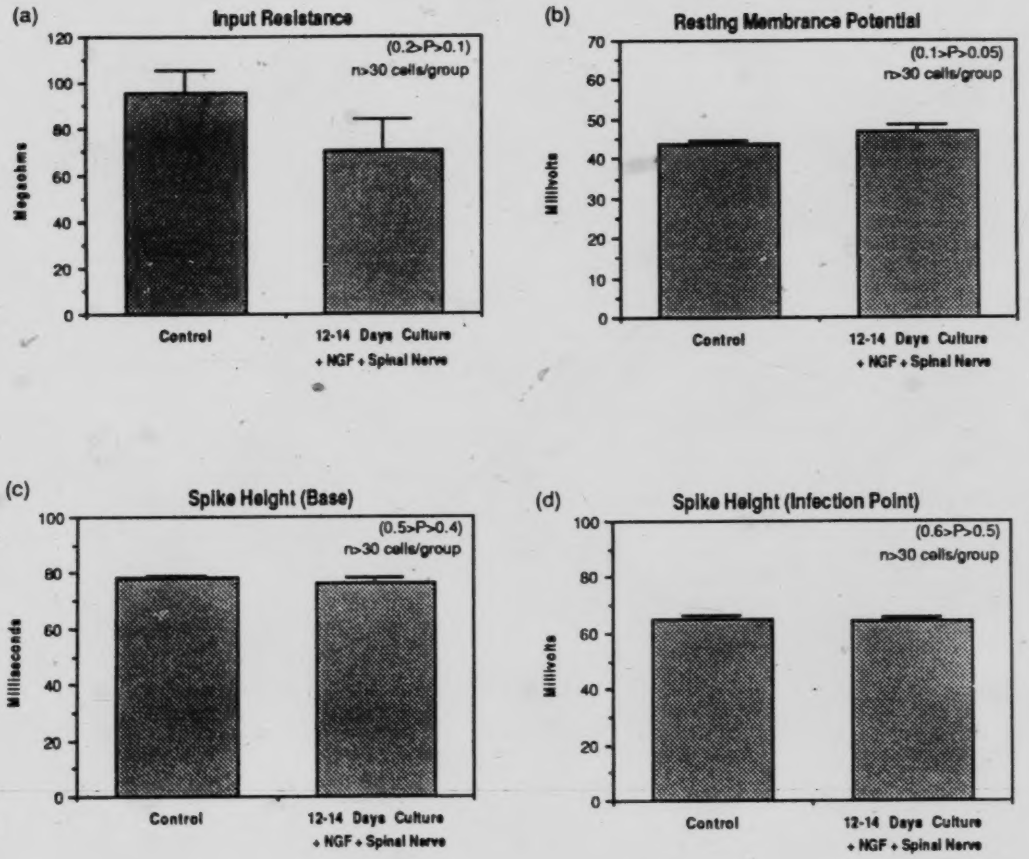


FIGURE 33:

EFFECT OF AXON LENGTH AND EXOGENOUS NERVE GROWTH FACTOR ADDITION ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF EXPLANTED BULLFROG SYMPATHETIC GANGLIA (OTHER PARAMETERS).

CONDITIONS ON THE MORPHOLOGY OF BULLFROG SYMPATHETIC GANGLIA EXPLANT CULTURES.

All post-synaptic BFSG neurones in explant culture were axotomized as a consequence of excision from the animal, and were also maintained in the absence of any target organs. Therefore, the observed morphological changes are those occuring as a consequence of axotomy and target removal, and also due to alterations in the composition of the culture media.

A. Morphology of Explant Cultures in Standard Culture Medium :

In this condition, neurones remained healthy for experimental durations up to 30 days in culture (as determined by electrophysiological examinations). The neuritic outgrowth from the explant was minimal in the standard culture medium, (refer to plate 2A). Plate 2A shows a 12 day old explant culture of a BFSG in which only a few neurites have sprouted. In extended culture durations of 20 to 30 days, there was only a slight enhancement of neurite sprouting (refer to plate 2B). Plate 2B displays a 25 day old explant of a BFSG.

It was also observed that neurites which sprouted from ganglia explanted in standard culture conditions remained

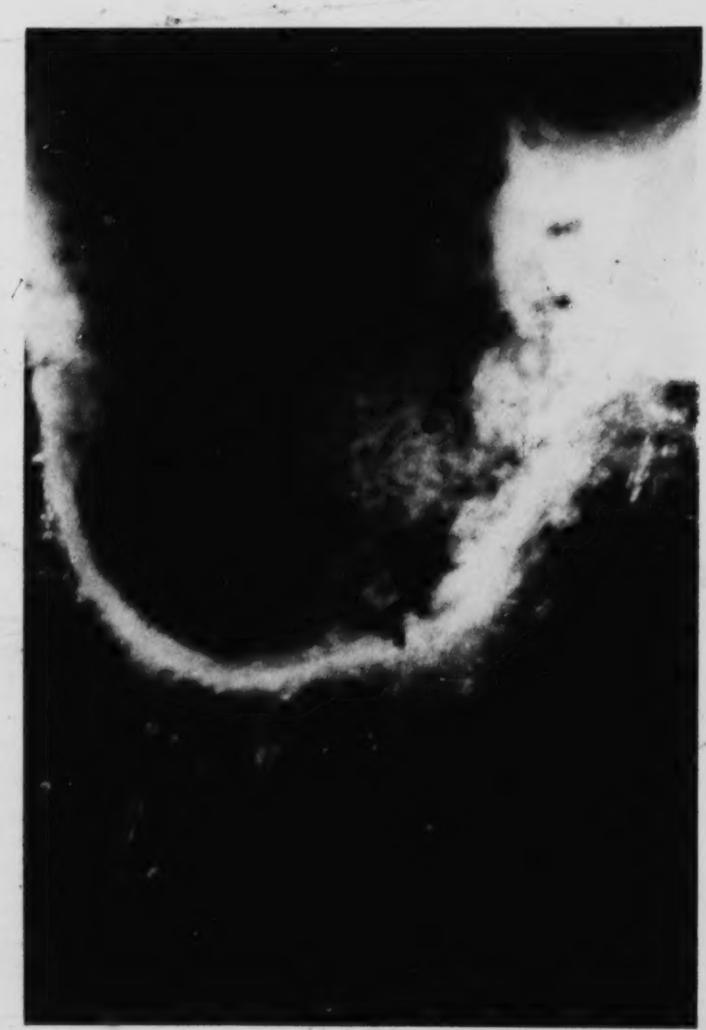


PLATE 2A :
EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN
STANDARD CULTURE MEDIUM (12 DAYS) MAGNIFICATION 100X's

PLATE 2B.:

EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD MEDIUM (25 DAYS) MAGNIFICATION 100X's.

small in diameter and immature relative to neurites examined in other culture conditions which will be discussed later, (refer to plate 2C).

Therefore, the time that the ganglion is maintained in the standard culture medium, in the absence of any target organs, determines the number of neurites which sprout from the explanted ganglion. Also, neurites remain small in diameter and developed slowly under these conditions.

B. Morphology of Explant Cultures in Standard Culture Medium Containing Nerve Growth Factor.

Explant cultures of BFSG neurones were maintained in standard culture medium identical to the previous condition, except for the addition of mouse salivary gland 2.5s NGF (50ng/ml).

The addition of NGF to the culture medium bathing the explant induces a dramatic increase in both the number and length of neurites sprouting from the ganglion (refer to plates 3a and 3b). If plates 3a and 3b are compared to plates 2a and 2b it can be observed that in the absence of targets and in the presence of NGF, neurites sprouting from 12 day old ganglion are increased in number and are greatly elongated. This type of neuritic morphology is not evident in either the 12 day or 25 day old explants (plates 2a and

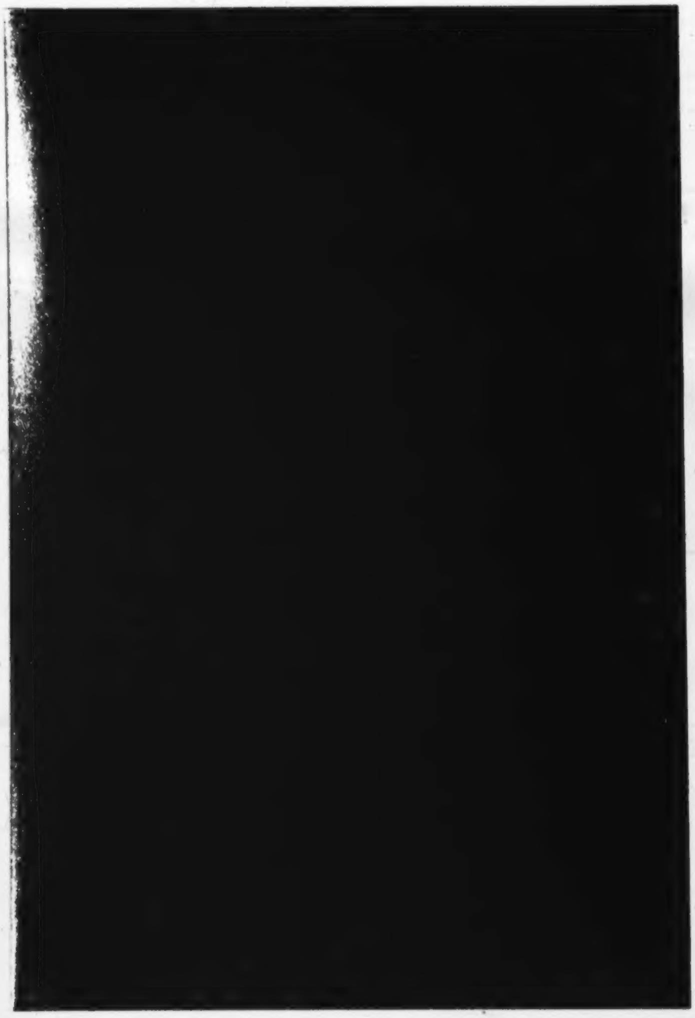


PLATE 2C :
NEURITE EXTENDED FROM AN EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD CULTURE MEDIUM (25 DAYS). MAGNIFICATION 400X's.

PLATE 3A :
EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD CULTURE MEDIUM PLUS NGF (50 ng/ml) FOR A 12 DAY PERIOD. MAGNIFICATION 100X's.

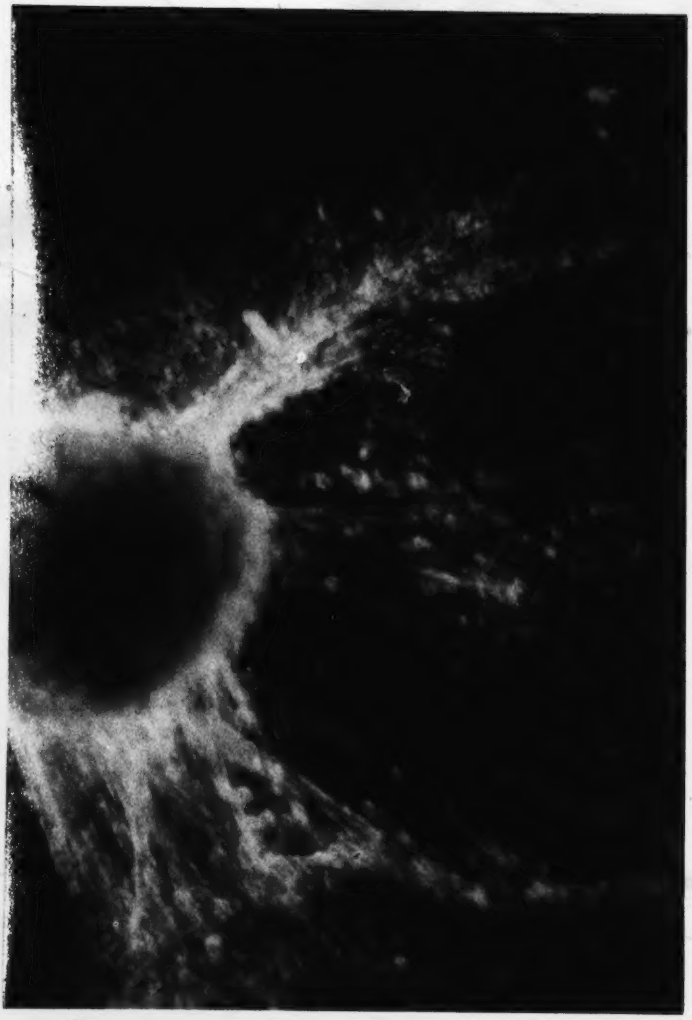


PLATE 3B :

EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD CULTURE MEDIUM PLUS NGF (50ng/ml) FOR A 12 DAY PERIOD. MAGNIFICATION 100X's.

2b) which were not exposed to NGF.

Also, examinations of high power micrographs of neurites sprouting from explants maintained in the presence of NGF (refer to plate 3c) displayed thickened, highly nodular processes. When plates 2c and 3c are compared it is obvious that neurites having access to NGF have reached a much higher level of maturation, or increased fasciculation of neurites is occuring.

Therefore, NGF is promoting outgrowth and enhancing the development of neurites extending from explanted adult BFSG neurones.

C. Morphology of Explant Cultures in Standard Culture Medium exposed to NGF for 24 Hours.

Explants were maintained for 11-13 days in standard culture medium prior to a 24h NGF (50 ng/ml) exposure. Plate 4 is a photomicrograph showing a 12 day old explant exposed to NGF for 24 hrs. Comparision of photomicrographs of neuritic sprouting and elongation between this condition and the two previous culture conditions shows that explants maintained in standard culture medium plus NGF displayed notable differences.

1. Explants in Standard Culture Conditions verses

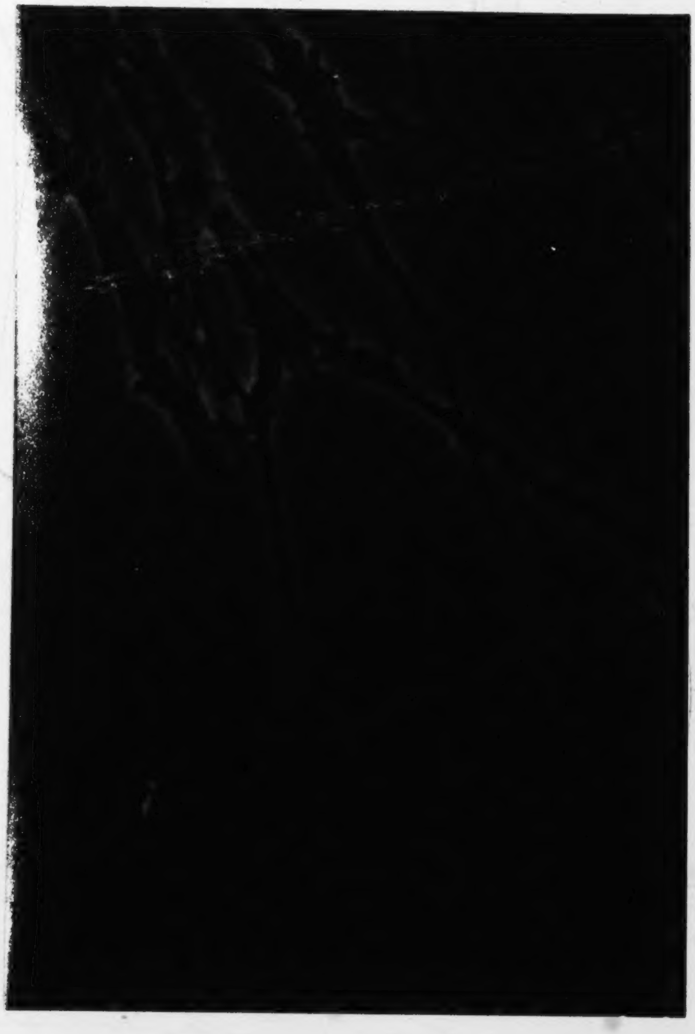


PLATE 3C :
NEURITE EXTENDED FROM AN EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD CULTURE MEDIUM PLUS NGF (50 ng/ml) FOR A 12 DAY PERIOD. MAGNIFICATION 400X'S.

PLATE 4 :
EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD MEDIUM FOR AN 11 DAY PERIOD THEN EXPOSED TO NGF (50ng/ml) FOR A 24 hr PERIOD.
MAGNIFICATION 100X'S.

Explants in Standard Culture Conditions Exposed to NGF for 24 hours

Comparision of photomicrographs of same age explants (Plates 2a and 4) easily demonstrates that the explant which received a 24 hour exposure to NGF displayed an enhanced neurite number and elongation.

2. Explants in Standard Culture Conditions plus NGF Verses Explants in Standard Culture Conditions Exposed to NGF for 24 Hours.

Comparision of same age explants (plates 3b and 4) demonstrates that the explants receiving constant exposure to NGF throughtout the culture duration produced a neuritic outgrowth and elongation that was greater than that produced by the explants receiving only a 24 hour NGF exposure.

Therefore, these experiments have shown that NGF induces a significant but submaximal amount of neurite outgrowth and maturation within a 24 hour period.

D. Morphology of Explant Cultures in Standard Culture Medium Containing Anti-NGF.

In this condition, explant cultures of BFSG were maintained in standard culture medium except for the addition of anti-NGF (affinity isolated sheep IgG, 0.5mg/ml).

Rather unexpectedly, the introduction of anti-NGF into the culture medium induced the sprouting of neurites from the explanted ganglia (refer to plate 5a). Neurite number and elongation was greater in explants maintained in standard medium plus anti-NGF than in standard medium alone (compare plates 2a and 5a). Neverthless, neuritic outgrowth was found to be far less than in same age explants which received constant NGF exposure throughout the culture duration (plate 3a verses plate 5a).

Although neurite number and elongation was enhanced in the presence of anti-NGF, neuritic maturation was minimal. Neurites produced in the presence of anti-NGF had a similar structure to those produced in standard culture medium alone (plate 2c vs plate 5b) and were noticably less developed than those neurites produced in the presence of NGF (plate 3c vs plate 5b).

Therefore, anti-NGF induced neurite extension from the explanted ganglia but neurite thickening did not occur.

1.Morphology of Explant Cultures in Standard Culture Medium containing NGF and anti-NGF:

When NGF (50ng/ml) and anti-NGF (.5ug/ml) were

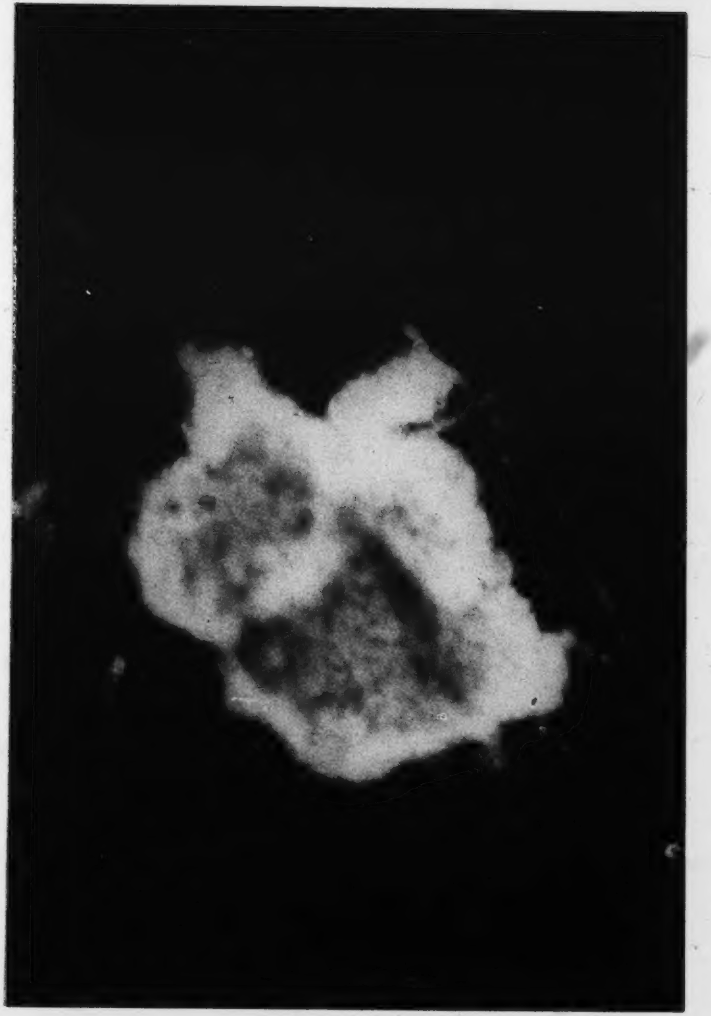


PLATE 5A :

EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD MEDIUM PLUS ANTI-NGF (0.5 μ/ml) FOR A 12 DAY DURATION. MAGNIFICATION 100X's.

PLATE 5B:

NEURITE EXTENDED FROM A 12 DAY OLD EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD CULTURE MEDIUM PLUS ANTI-NGF (0.5 μg/ml). MAGNIFICATION 400X's introduced simultaneously into the medium bathing the explanted ganglia, several morphological changes in the neuritic outgrowth were observed when comparisons were made with the morphology of explants maintained under different conditions.

Firstly, both fibre number and elongation was increased relative to the neuritic outgrowth produced by anti-NGF alone (plate 6a vs 5a), but was less than that produced by NGF alone (plate 6a vs 3b). Secondly, neurite maturation, or thickening in the presence of both NGF and anti-NGF was enhanced relative to the degree of maturation or development produced by anti-NGF alone (plate 6b vs 5b). Further, when a comparision between the anti-NGF plus NGF condition and the NGF alone condition was completed with respect to neurite maturation, no differences were readily observed (Plate 6b vs 3c).

Therefore, these observations suggest that when explants are exposed simultaneously to NGF and anti NGF, the effects of NGF with respect to neurite number and elongation are reduced but its effects on neuritic maturation remain normal.

E. Summary of the Effects of Changing Culture Conditions on Explant Cultures of BFSG.

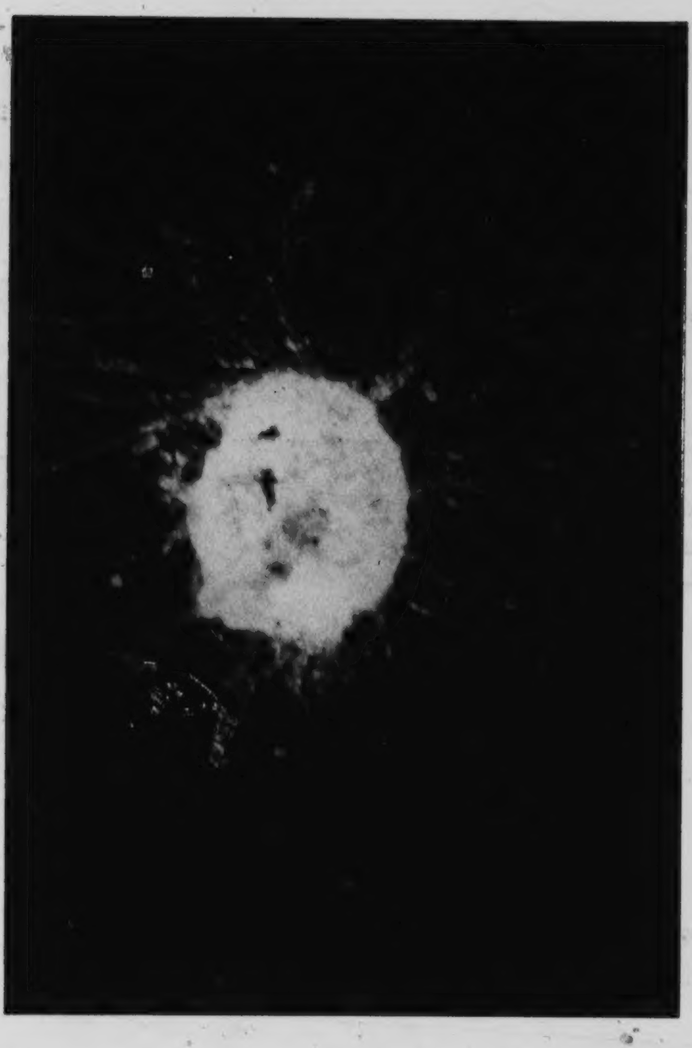


PLATE 6A :
EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD MEDIUM SUPPLEMENTED WITH NGF (50 ng/ml) PLUS ANTI-NGF (0.5 µg/ml) FOR A 12 DAY PERIOD. MAGNIFICATION 100X's.

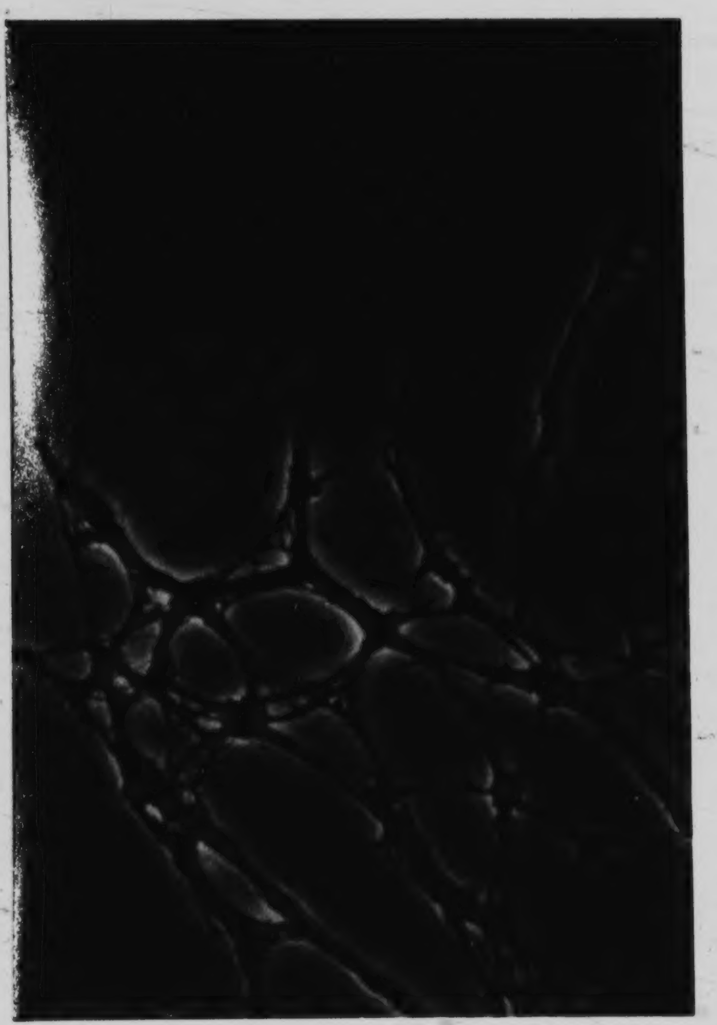


PLATE 6B :
NEURITIC OUTGROWTH FROM AN EXPLANTED BULLFROG SYMPATHETIC GANGLION MAINTAINED IN STANDARD MEDIUM SUPPLEMENTED WITH NGF (50 ng/ml) AND ANTI-NGF (0.5 µg/ml) FOR A 12 DAY PERIOD. MAGNIFICATION 400X's.

Explants maintained in standard culture medium remain healthy and neurites sprout from the post-synaptic sympathetic ganglionic neurones. These neurites are few in number and do not undergo thickening and possess few varicosities.

Explants maintained in standard culture medium plus NGF (50ng/ml), produce a large number of elongated neurites. These neurites mature and develop varicosities (Levi-Montalcini et al., 1985).

Explanted BFSG exposed to anti-NGF also produce neurites. The number of neurites produced is less than the number produced by explants exposed solely to NGF. Also, neurites sprouting in response to anti-NGF do not appear to undergo maturation (Hulsebosch et al., 1987).

When anti-NGF and NGF were introduced simultaneously into culture medium bathing explants of BFSG, neuritic outgrowth occured. Neuritic outgrowth was greater in this condition than with anti-NGF alone, but was less than NGF alone. Also, neurites sprouting in this condition underwent maturation comparable to the degree of maturation in the presence of NGF alone.

VIII. ELECTRON MICROSCOPIC STUDY OF THE CELL BODY RESPONSE OF BFSG NEURONES TO AXOTOMY AND CULTURE

DURATION.

In many types of nerves, transection of a nerve axon induces several morphological and metabolic changes in the associated cell body (Hyden, 1960; Leiberman, 1971). These changes are collectively referred to as chromatolysis (Marinesco, 1896). A previous study of the effect of axotomy on BFSG (Gordon et al., 1987) has shown that the electrophysiological changes occur in the absence of marked morphological in the cell body. It would therefore seem that electrophysiological changes represent an early response to target disconnection. In order to determine if chromatolytic reactions were occuring within the perikarya of explanted BFSG neurones maintained in standard culture medium, in the absence of target organs, an electron microscopic examination of the cell bodies of these neurones was performed.

A Philips 410 electron microscope was used to study the cell bodies of control BFSG just after removal from the animal, and 12 day old explants of BFSG. All cells were examined under a magnification of 9240x's (plates 7a and 7b). The criteria used to determine if chromatolysis was induced as a consequence of axon injury were as follows (Lieberman, 1971).

1) Migration of the neucleus to an eccentric position within the cell.

PLATE 7A
ELECTRONMICROGRAPH OF THE CELL BODY OF AN ACUTELY EXCISED BULLFROG SYMPATHETIC GANGLION NEURONE.
MAGNIFICATION 9240X's.

PLATE 7B
ELECTRONMICROGRAPH OF THE CELL BODY OF AN EXPLANTED BULLFROG SYMPATHETIC GANGLION NEURONE MAINTAINED IN STANDARD MEDIUM FOR A 12 DAY PERIOD.
NOTE : SECTION DOES NOT PERMIT OBSERVATION OF THE NUCLEUS.

LEGEND: PLATES 7A AND 7B

N = NUCLEUS
n = NUCLEOLUS

RE = ROUGH ENDOPLASMIC RETICULUM

m = MITOCHONDRIA

P = PIGMENT GRANULE (LYSOSOME)

Both cells possess rough endoplasmic reticulum, the nuclei of both conditions remain of similar size and position, and no nucleolar vacuolarization was present. Furthermore, the positioning of the nuclei in both conditions was identical.

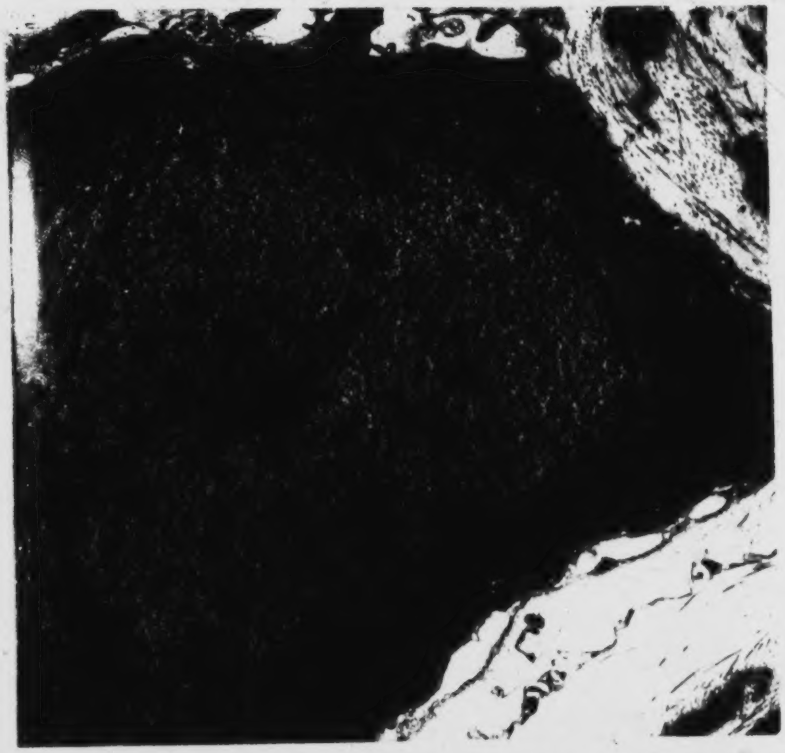


Plate 7a

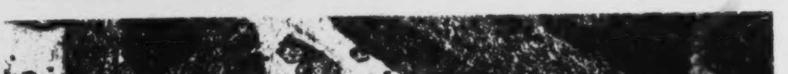


Plate 7b

2) Increased size, vacuolation and eccentricity of the nucleoli.

3) A disintegration of the large concentrations of granular endoplasmic reticulum (nissl bodies) throughout the cell.

Analysis of electronmicrographs of approximately 20 cell bodies from each condition demonstrated that with respect to the above criteria, no differences in cell body morphology existed between the control ganglia and the explanted ganglia (plates 7a and 7b). Therefore, this strictly morphological investigation of the perikaryal response of BFSG neurones to axotomy, suggests that chromatolysis does not occur in BFSG neurones in response to axotomy, target deprivation and culture duration.

DISCUSSION

I. DOES NGF REGULATE THE ELECTROPHYSIOLOGICAL PROPERTIES OF BULLFROG SYMPATHETIC GANGLION NEURONES?

The results of this investigation support the hypothesis that NGF is involved in the maintenance of specific AP parameters in bullfrog sympathetic ganglion neurones. Furthermore, morphological and electrophysiological data suggest that the magnitude of spike width and AHP amplitude are correlated with growth (production of neurites), whereas the magnitude of the AHP duration is closely regulated by accessibility to NGF. This study also provides evidence which supports the existence of endogenous NGF in Rana Catesbeiana

The characteristic electrophysiological properties of BFSG neurones maintained in each culture condition was dependent upon the composition of the culture medium, the presence of NGF or anti-NGF, the length of the severed axon and the duration of NGF treatment. These factors also determined the extent of neurite outgrowth or axon regeneration. Consequently, in the following sections possible explanations for the electrophysiological responses and morphological changes induced by different culture conditions will be discussed.

A. Electrophysiological characteristics of control neurones.

Electrophysiological examination of acute preparations of BFSG bathed in standard culture medium, demonstrated that the APs produced by B-cells in response to the injection of a brief depolarizing current, were almost identical with respect to measured AP parameters to recorded previously in vivo from bullfrog sympathetic ganglia B-cells by other workers (Kelly et al., 1986; Gordon et al., 1987). All control cells were primarily characterized by a short spike duration ranging between 1-2 msec, a large AHP amplitude (17-20 mV) and a long AHP duration (250-300 ms), (see Table 3).

The two component AHP of BFSG neurones accounts for these particular characteristics (Pennefather et al., 1985). The AP repolarizes quickly and has a large AHP amplitude due to the presence of a rapidly activating, voltage sensitive, Ca^{2+} sensitive K^{+} current, termed I_C, (Adams et al., 1982b) while the extended AHP duration is due to the activation of a slowly activating voltage insensitive, Ca^{2+} sensitive K^{+} current (I_{AHP}; Pennefather et al., 1985; Goh and Pennefather 1987; Lancaster and Pennefather 1987). Influx of Ca^{2+} through voltage-sensitive Ca^{2+} channels during and after the "spike phase" of the AP is sufficiently rapid to trigger the activation of these Ca^{2+} sensitive K^+ channels, which in turn serve to hyperpolarize the membrane. The classical Ca^{2+} insensitive delayed rectifier K^+ current activates too slowly to participate in AP repolarization or AHP generation (Goh and Pennefather, 1987).

The extent of neuronal injury following microelectrode penetration may be an important determinant of the observed AHP duration (Kuba et al., 1983; Gordon et al., 1987). Therefore, only those cells which generated APs with spike heights greater than 70mV and had RMPs between -45mV and -60mV were recorded and used in data interpretation. This minimized the possibility that the changes in AHP characteristics were due to penetration injury (see Gordon et al., 1987).

In this study, electrophysiological recordings from acutely excised BFSG were used as a standard to which all other electrical responses from explanted sympathetic ganglion neurones maintained under different conditions were compared.

B. Electrophysiological characteristics of explanted bullfrog sympathetic ganglia maintained in standard medium.

Examination of the electrophysiological properties of explanted BFSG neurones has shown that axotomy and target removal produces a reduction in AHP duration and amplitude, an enhancement of spike duration and an increase in rheobase. These changes, which are not attributable to alterations in input resistance or RMP are similar to those seen in axotomized BFSG neurones in vivo (Kelly et al., 1986; Gordon et al., 1987; Shapiro et al., 1987), (Figs 8 - 13)

It is important to note that explanted BFSG neurones have also been denervated (ie. preganglionic fibres have also been transected during removal of the ganglion from the animal). However, these results demonstrate that explanted BFSG neurones undergo similar axotomy-induced electrophysiological responses to axotomy as do axotomized neurones in vivo, which do not have their preganglionic inputs severed. Furthermore, other work from this laboratory (K.K. Pant, T. Gordon, and P.A. Smith, unpublished observations) has demonstrated that dennervation of BFSG neurones does not alter the shape of the AP of BFSG neurones.

Studies of the time course of the electrophysiological changes that occur within target deprived BFSG neurones demonstrate that AHP duration falls to a minimum value within 1 to 2 days culture duration, whereas, AHP amplitude falls exponentially to a minimum value over a period of ten days. Spike duration continues to increase progressively as time in culture increased, while rheobase peaked after about 8 days and then returned to control levels. The different rates at which these parameters change in response to target deprivation suggests that different processes may be involved in each case.

The changes seen in AHP parameters and spike width in explanted neurones as a consequence of axotomy and target deprivation may be due to a functional loss of both types of Ca^{2+} sensitive K^+ channels. A functional loss of these channels may be due to several factors. One possible explanation would be an alteration in Ca^{2+} influx during depolarization of the membrane. If Ca^{2+} influx was decreased, spike duration would increase, and AHP amplitude and duration would decrease, due to a functional loss of both I_C and I_{AHP}. However, this is unlikely as experiments by Kelly et al. (1986) have demonstrated that regenerative Ca^{2+} spikes can be generated in both control and axotomized neurones. Alterations in the sequesteration of neuronal Ca^{2+} may also result in a functional loss of Ca^{2+} sensitive K^+ conductance which would also account for the electrophysiological response to target deprivation (Kelly et al., 1986). Alternatively, the production of membrane properties characteristic of axotomized neurones (Chalazonitis et al 1987) could result from a modulation of the functional state of these channels as a result of changes in proteins which control their gating mechanisms. Another explanation could be that a decrease in the number of Ca^{2+} sensitive K^+ occurs.

Changes in spike duration could also be attributed to changes in Na^+ channel inactivation. Changes in the kinetics of activation of these channels could contribute to the changes in rheobase. Therefore, in order to accurately determine which possible mechanism is responsible for the alterations in the membrane electrical properties that occur after target deprivation, voltage clamp and patch clamp studies would be necessary. Voltage or whole cell patch clamp studies would allow for the separation of I_C and I_{AHP}, analysis of the kinetics of Ca^{2+} influx, and the investigation of any alterations in sodium currents (I_{Na}), while single channel studies would enable the study of the kinetics, conductance and number of I_C channels. It is perhaps significant that AHP duration at 75% repolarization is much less affected by target deprivation than that at 100%, despite the inherent inaccuracies in measuring AHP duration in this way. At the longer time period, I_{AHP} would be the only K^+ current operating, so these data suggest that I_{AHP} is clearly influenced by target deprivation.

Another interesting finding was that the APs generated by target deprived or axotomized neurones in culture, resemble those seen in developing spinal neurones dissociated from Xenopus neural plates. These neurones exhibit long duration APs and do not develop Ca^{2+} sensitive K^{+} conductance until late in development (Spitzer and Lamborghini, 1976; Blair, 1983; Blair and Dionne, 1983; O'Dowd, 1983). This similarity between target-deprived and embryonic neurones supports the hypothesis that axon transection may induce a differentiation towards an embryonic stage which is more condusive to growth and axon regeneration (Kuno et al., 1974, Grafstein, 1977; Gordon, 1983; Gordon et al., 1987). Axotomy-induced differentiation may promote a functional loss of Ca^{2+} sensitive K^{+} channels, which would result in the prolonged AP depolarization characteristic of embryological neural tissue (Blair and Dionne, 1983).

Also, since neural regeneration has been linked to an increased intracellular Ca^{2+} concentration it is possible that a shift towards an embryonic state may promote regeneration (Linas, 1979; Grinvald et al., 1981; Suarez Isla et al., 1984; Kelly et al., 1986).

Electron microscopic analysis of the cell bodies of 12 day old explants of BFSG maintained in standard medium, demonstrated that these cells did not undergo chromatolysis in response to axotomy and target removal.

Therefore in the amphibian, target removal and axotomy promoted electrophysiological changes but did not induce any corresponding changes in cell body morphology. Consequently, this may signify that the axotomy-induced electrophysiological changes are more fundamental with respect to axon regeneration in the bullfrog, than is the cell body response to axon transection and target removal.

C. Electrophysiological characteristics of explanted bullfrog sympathetic ganglia maintained in standard medium plus NGF.

It has been demonstrated that mechanical damage itself is not responsible for the observed electrophysiological changes which accompany axotomy of dorsal root ganglion neurones (Gallego et al., 1987). Therefore, loss of continuity between the cell body and target organ may cause a loss of trophic support from the target which may mediate the observed changes in membrane properties (Czeh et al., 1977 ; Gordon, 1983; Gallego et al., 1987). NGF has been shown to induce the differentiation of pheochromocytoma cells, (Greene and Shoter, 1980), increase ion channel number (Reed et al 1986; Rudy et al., 1987) and alter ion channel sensitivity to specific channel blockers (Takahashi et al, 1985). Thus, the number or possibly the function, of the ion channels in BFSG neurones which determine spike width, rheobase, and AHP amplitude and duration, may be dependent upon the constant retrograde supply of NGF from the target organ. This hypothesis was tested by introducing NGF into the standard culture medium bathing BFSG explants.

Investigation of the membrane properties of explanted BFSG, maintained in standard medium plus NGF, demonstrated that NGF can prevent the occurrence of some (but not all) of the electrophysiological changes produced by axotomy and the resulting discontinuity of the cell body with its target. NGF application successfully prevented the axotomy-induced reduction in AHP duration, however, AHP amplitude was not completely restored to control levels, but was significantly greater than AHP amplitude measured in the absence of NGF (Figs 8, 18 - 20). Increases in rheobase were also prevented by NGF (Fig 22). In contrast however, NGF induced a significant and progressive increase in spike width (Fig 21).

Therefore, these findings suggest that, the AHP duration of BFSG neurones appears to be strictly dependent upon access to NGF. Chalazonitis et al.,(1987) has suggested that NGF may modulate the functional state of existing Ca^{2+} sensitive K^+ channels via changes in proteins regulating the gating mechanism of these channels. Thus NGF may be required for the normal activity of slowly activating voltage insensitive, Ca^{2+} sensitive

K^+ channels.

The presence of NGF in the culture medium of explanted BFSG neurones did not totally prevent the axotomy induced decrease in AHP amplitude. Fast activating, voltage sensitive, Ca2+ sensitive K+ channels, which aid in AP repolarization and determine spike width and AHP amplitude, may therefore be less dependent on accessibility to NGF. This is supported by the finding that the AHP amplitude of explanted BFSG decreased more slowly than AHP duration as time in culture in the absence of NGF increased. Therefore, the effects of axotomy and target deprivation were less severe with respect to AHP amplitude than with respect to AHP duration. If this is the case, AHP amplitude may eventually return to control levels, in the presence of NGF but at a much slower rate than does AHP duration (Kelly et al., 1988). Another possibility is that I_C is partially dependent upon another unknown trophic factor produced by the target, exposure to NGF alone will not restore I_C activity to control levels. As a result, full recovery of AHP amplitude and a reduction of spike duration will not occur until reinnervation of the target is completed, and accessibility to the factor is re-established. This effect may even require functional re-innervation (Kelly et al., 1988).

NGF is also known to increase the number of functional Na+ channels, TTX resistant Na^+ channels and brain type II Na^+ channels in pheochromocytoma cells (Dichter et al 1977; Reed et al, 1986; Rudy et al.,1987; Mandel et al., 1988). Therefore changes in Na+ channels may account for the increase in spike width and for the observation that the value of rheobase determined for 12 day old explant cultures of BFSG maintained in the presence of NGF did not differ significantly from control values, while the rheobase value of 12 day old explants maintained in the absence of NGF was significantly enhanced relative to control.

An electron microscopic examination of the cell bodies of neurones exposed to NGF in vitro was not performed. The reason for this decision was based on the fact that the neuronal cell bodies of explanted BFSG maintained in standard medium in the absence of target cells did not differ morphologically from control cells. Therefore because axotomy and target removal did not induce chromatolysis, observation of morphological differences between these cells and NGF-treated cells would not be noticable. However, due to the fact that each of these conditions produces changes in electrophysiological properties it may be that NGF exerts its effects directly upon the ion channels via a phosphorylation mechanism, (Cremins et al., 1986) rather than acting at the level of the necleus and inducing protein synthesis (Thoenen and

Barde, 1980).

Obviously, a more accurate interpretation of the NGF-induced changes in explanted BFSG neurone ion channel number and properties is required. Therefore the current clamp experiments performed in this study, represent a starting point for further electrophysiological analysis.

D. Examination of the electrophysiological characteristics of explanted BFSG neurones maintained in standard medium plus anti-NGF.

Electrophysiological analysis of BFSG explants maintained in medium containing Anti-NGF demonstrated that spike duration is enhanced to a value significantly greater than that produced in any other experimental culture condition (Figs 8 and 21). The AHP amplitude is reduced, (Fig 20) and is significantly less than the reduction induced by axotomy and target removal alone. Measured values of AHP duration are similar to the AHP duration of explanted BFSG neurones maintained in standard medium (Figs 18 and 19). These observed changes demonstrate that with respect to some AP parameters, anti-NGF enhances the effects of axotomy and target removal.

In those explants maintained in standard medium alone, enhancement of spike width and the reduction in AHP parameters was attributed to loss of access to target derived NGF or other factors. The introduction of anti-NGF to BFSG explants serves to neutralize all possible sources of endogenous NGF i.e. NGF derived from glial tissue surrounding the neurones of the explanted ganglion (Abrahamson et al., 1986; Assouline et al., 1987). Therefore, the observed enhancement of the axotomy-induced electrophysiological changes may be explained in terms of a more pronounced loss or functional alteration of both types of Ca^{2+} sensitive K^+ channels, which would result in a dramatic increase in spike width. Therefore, the anti-NGF induced depletion of endogenous NGF may further dedifferentiate the neurones of explanted BFSG. This possible dedifferentiation is exemplified by a prolonged spike duration and a further reduction in AHP amplitude; electrophysiological properties characteristic of developing Xenopus embryonic spinal neurones (Spitzer and Lamborghini,1976; Blair,1983; O'Dowd,1983; Blair and Dionne, 1985). Thus, prolonged spike duration enhances Ca^{2+} ion entry during depolarization of the membrane potential. Therefore, more Ca^{2+} will enter neurones of explanted BFSG maintained in standard medium plus anti-NGF in comparison to the neurones of BFSG explants maintained in standard medium alone. Increased internal Ca^{2+} has been linked to neuronal regeneration (Grinvald et al 1981, Suarez-Isla et al 1984). Consequently, this should be represented as an enhanced neurite outgrowth (i.e.

regeneration) from explants receiving anti-NGF treatment.

Morphological examination of explants exposed to anti-NGF demonstrated that the neuritic outgrowth from these expants was superior with respect to number and length to the neuritic outgrowth observed from explants maintained in standard medium (Plates 2a and 2b vs 5a).

Another possible explanation for this enhanced neurite outgrowth in the presence of anti-NGF, is that retrogradedly transported NGF serves to signal that the cell is still attached to its appropriate peripheral target. Thus if NGF is removed (i.e. anti-NGF application and target removal) neuronal cell bodies may "interpret" this as a sign of axon detachments and begin sprouting (Hulsebosch et al., 1987).

II. INTERPRETATION OF THE RESULTS OF OTHER EXPERIMENTS.

A. Proof of the effectiveness of NGF antibodies:

In order to determine if anti-NGF (affinity isolated sheep IgG) is effective in neutralizing exogenously applied mouse 2.5s beta NGF, the electrophysiological properties of explanted bullfrog sympathetic ganglion neurones maintained in standard medium supplemented with NGF plus anti-NGF, were examined. The results of this study demonstrated that the electrophysiological effects of NGF and anti-NGF with respect to AHP amplitude and duration, and spike width were significantly reduced in explants maintained in NGF plus anti-NGF simultaneously (Fig 8, 18 - 21).

Therefore, two major conclusions can be drawn. Firstly, that anti-NGF can be successfully introduced into the culture medium and was not lost during the filtration/sterilization procedure. Secondly, the anti-NGF used in this study is active and effectively neutralizes mouse salivary gland NGF.

B. Time course of the effects of NGF.

A 24 hour NGF exposure tended to reverse the decrease in AHP duration and amplitude produced by axotomy, target removal, and extended culture duration (Fig 27a to 27c). Also neurite outgrowth from explants receiving the 24 hour NGF treatment was more extensive than that produced by same age cultures which did not recieve NGF treatment. NGF can therefore induce a change in the electrophysiological properties of explanted BFSG neurones, and enhance neurite outgrowth within a short period of time.

NGF may directly induce ion channel synthesis which would account for these changes. Much evidence exists which demonstrates that NGF rapidly induces protein synthesis (Thoenen and Barde, 1980), but most studies of ion channel induction by NGF, involve NGF treatment of PC 12 cells for several days before the examination of ion channel number was undertaken, (Reed and England, 1986; Rudy et al., 1987; Mandel et al., 1988). Therefore, the exact time course of NGF induced ion channel production appears to be unknown.

Another possible mechanism by which NGF may initiate a reversal of the axotomy induced electrophysiological changes may be via the activation of second-messenger systems. In PC12 cells, NGF has been shown to mediate the activation of both CAMP dependent protein kinase and C-kinase, which in turn phosphorylate substrate proteins (Cremin et al, 1986). Therefore, these substrate proteins may include the proteins involved in regulating the gating mechanisms of specific ion channels. Therefore, activation phosphorylation of ion channels, would explain the rapid NGF induced change in the electrophysiological properties of explanted BFSG.

C. Axon length and the electrophysiological properties of BFSG neurones.

Distal transection of spinal nerves delayed the onset of the electrophysiological changes which occur when the axons of the paravertebral sympathetic ganglia are severed at the rami communicantes. The measured AHP duration at 100% and 75% was of a greater magnitude when the elongated cut axons remained attached to the neurone cell bodies (Fig 30a and 30b), and the rheobase values of cells maintained under these conditions were similar to those of control (Fig 30e). Perhaps the most interesting observation relating to explants with attached postganglionic axons was that only in this situation was there any reduction in the increase in spike width seen with all other tissue culture procedures (Fig 30d).

The delay of the onset of the axotomy-induced reduction in AHP parameters may be due to the presence of NGF within the severed axon which was being retrogradedly transported from the target prior to axotomy. Also the presence of glial tissue on the severed spinal nerve associated with the ganglia may also produce NGF. Therefore, retrogradely transported NGF and a greater quantity of glial derived NGF constitute two enogenous sources of NGF which explants of BFSG in which the spinal nerve has been completely removed do not have access to. Nevertheless, NGF always enhances spike width, therefore, the maintainance of a short spike width may be dependent on the presence of the axon per se or perhaps to certain quantities of other unknown retrogradely transported trophic substances which delay the loss or functional loss of specific ion channels which determine AHP duration and AP repolarization.

Although none of the parameter changes characteristic of axotomy could be completely prevented by the presence of spinal nerves plus NGF (Fig 31), the effects of axotomy were noticeably countered and most parameters were tending towards control values. One exception to this is spike duration, which was enhanced as a result of exogenous NGF application. This may be due to effects on Na+ channels as previously discussed.

Therefore, these experiments provide indirect evidence showing that NGF is retrogradely transported and produced by glial tissue in explant cultures. Further, even in the presence of exogenous NGF and attached spinal nerves, the electrophysiological parameters of axotomized BF[illegible]G do not recover to control values. This may suggest the requirement of another unknown trophic factor derived from the previously innervated target organ.

III. CORRELATION OF MORPHOLOGICAL AND ELECTROPHYSIOLOGICAL CHANGES.

Extensive neurite outgrowth occurs from explants which are maintained in standard medium plus NGF. Therefore, the NGF induced enhancement of spike width may correlate with a regenerative electrophysiological response, enabling increased Ca^{2+} influx which may aid in regeneration (Llinas, 1979; Greene and Shooter, 1980; Grinvald and Farber, 1981; Suarez Isla, 1984). Also, due to the fact that no targets are present within the culture for the extending axons to innervate, APs remain wide, despite the presence of NGF, and the neurones appear to remain in a growing or "seeking" mode (Gordon et al., 1983).

IV. Amphibian NGF.

It is known that different tissues in mammals, reptiles, birds, and fish produce proteins with NGF-like properties (Thoenen and barde ,1980; Murphy et al., 1984). Also a few of these NGF-like molecules have been shown to react with antibodies to mouse salivary gland NGF (Murphy et al., 1984). Therefore, there is evidence for the conservation of NGF structure and action across different animal species.

Thus, the enhancement of axotomy-induced electrophysiological changes by anti-NGF, the reduction of the electrophysiological effects of exogenously applied NGF by anti-NGF, and the evidence for the conservation of structure and function across several species, gives support for the hypothesis of this study that an endogenous

NGF-like molecule similar to mouse salivary gland NGF is produced by Rana catesbeiana. This endogenous factor is produced by glial tissue surrounding BFSG neurones and possibly by other target tissues. Furthermore, this factor is utilized by neurones and may be involved in maintaining the normal electrophysiological functioning of these cells.

V. Future Experiments.

A. Biophysical studies:

It is important to state that macroscopic examinations of membrane responses to injected depolarizing current via current clamp procedures are limited with respect to the degree to which one can determine what events are occuring at the ion channel level. Therefore, in order to further understand the molecular mechanism of axotomy and NGF-induced electrophysiological changes, voltage or whole cell patch clamp and single channel recording procedures are clearly required. Nevertheless, patch clamp studies are primarily performed on dissociated cells, therefore examination of BFSG electrophysiological properties would be performed on dissociated cells, which may differ electrophysiologically from explanted BFSG. Furthermore, explants serve to mimic the in vivo system, while study of dissociated ganglion neurones is further removed from the natural in vivo system.

Another important point to consider is that in whole cell-patch clamping the intracellular fluid of the cell is freely interchangable with the intracellular medium in the micropipette. Therefore, this may result in "wash-out" of important intracellular constituents which may regulate ion-channel functioning. Thus an incorrect interpretation of electrophysiological properties may result.

However, in order to understand the basic biophysics of these changes these studies are necessary.

B. Introduction of Target Organs.

Throughout this investigation all explants were maintained in the absence of target organs. The purpose of this was to control the immediate cellular environment of the explant, by eliminating the possible secretion of trophic factors by target cells. Therefore, the constituents of the culture medium was known at all times, and the electrophysiological effects of exogenously applied NGF on BFSG neurones could be examined in isolation.

Now that the electrophysiological effects of axotomy and target removal, NGF and anti NGF have been documented with respect to BFSG neurones, it would be interesting to determine if complete recovery of all AP parameters could be mediated by allowing regeneration of axons and target innervation to occur *in vitro*. NGF application alone could not re-establich the production of APs identical to control with respect to every AP parameter. Therefore, including target tissue in the *in vitro* environment of the explants would allow for the investigation of the possibility that the normal electrophysiological functioning of these neurones is dependent upon other possible trophic factors or synaptic contact and neurotransmitter secretion (Gordon 1983).

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